The Endothelium: An Essential Barrier Between Chronic Stress and Vascular Pathology

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Declaration

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Signature: Date: December 2019

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List of Abbreviations

Abbreviation	Meaning
Ab	Antibodies
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
ANGII	Angiotensin II
ANOVA	Analysis of variance
cAMP	Cyclic adenosine monophosphate
CD	Conjugated diene
CRH	Corticotropic-releasing hormone
CRHRH1	CRH receptor-1
CRHRH2	CRH receptor-2
CVD	Cardiovascular disease
DETAPAC	Diethylenetriaminepentaacetic acid
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
E	Epinephrine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELS	Early-life stress
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FMD	Flow-mediated dilation
GPx	Glutathione peroxidase
GR	Glucocorticoid receptor
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
KH	Krebs-Henseleit

LC-NE	Locus coeruleus-norepinephrine
LDL	Low-density lipoprotein
MESA	Multi-ethnic Study of Atherosclerosis
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
O_2	Oxygen
OH	Hydroxyl radical
$ONOO^-$	Peroxynitrite
ORAC	Oxygen radical absorbance capacity
Phe	Phenylephrine
PTSD	Post-traumatic stress disorder
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
SA	South Africa
SEM	Standard error of the mean
SNS	Sympathetic nervous system
SO	Superoxide
SOD	Superoxide dismutase
sTF	Soluble tissue factor
TBARS	Thiobarbituric acid
TNF-α	Tumour necrosis factor-alpha
U	Enzyme units
UCMS	Unpredictable chronic mild stress
VCAM-1	Vascular cell adhesion molecule-1
VWF	Von Willebrand factor

Foreword

This dissertation is presented in article-format. The first chapter serves as a review of literature relevant to the thesis topic, while the second chapter reports on results obtained from various experimental analyses conducted during the degree.

Chapter 1:

The Endothelium: An Essential Barrier Between Chronic Stress and Vascular Pathology

Abstract

In recent years, chronic stress has emerged as an influential and understudied risk factor for the onset of cardiovascular disease. While much is known regarding the physiological systems that orchestrate the innate stress response, limited knowledge exists regarding the molecular derangements that underpin cardiovascular pathologies. Endothelial integrity is essential for maintaining a stable internal environment. Stress-mediated cardiovascular, metabolic and immunologic alterations negatively impact the vaso-reactive capabilities of the endothelium. Furthermore, chronically elevated circulating levels of glucocorticoids and catecholamines not only directly influence nitric oxide availability but further contribute towards a proinflammatory and prooxidative state. A dysfunctional endothelial layer in turn facilitates the development of stenotic and atherosclerotic vascular lesions. This review highlights the current burden of chronic stress globally and in South Africa. We subsequently discuss the activity and regulation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system following exposure to acute and chronic stressors. Lastly, we focus on mechanistic avenues that contribute towards endothelial dysfunction and a compromised cardiovascular system.

Opsomming

Chroniese stres het onlangs as ’n belangrike risiko-faktor vir kardiovaskulêre siektes aan lig gekom. Alhoewel daar reeds heelwat kennis bestaan oor die fisiologiese stelsels wat die stresrespons reguleer, is daar beperkte kennis aangaande die molekulêre afwykings van kardiovaskulêre patologie. Endoteel integriteit is noodsaaklik vir die handhawing van 'n stabiele interne omgewing. Stres-gemedieërde kardiovaskulêre, metaboliese en immunologiese veranderinge het 'n negatiewe invloed op die vaso-reaktiewe vermoëns van die endoteel. Verder beïnvloed chronies verhoogde vlakke van glukokortikoïede en katkolamiene in die sirkulasie nie net die beskikbaarheid van stikstofoksied nie, maar dra dit ook tot 'n pro-inflammatoriese en pro-oksidatiewe toestand by. Op sy beurt fasiliteer ’n disfunksionele endoteel die ontwikkeling van stenotiese- en aterosklerotiese vaskulêre letsels. Hierdie resensie beklemtoon die invloed van chroniese stres op Suid-Afrika. Hieropeenvolgend, word die werking en regulering van die hipotalamus-pituïtêre-bynier-as en die simpatiese senuweestelsel na die blootstelling aan akute en chroniese stressors bespreek. Ten slotte word daar op die meganistiese weë wat tot die endoteel wanfunksie en kardiovaskulêre probleme bydra, gefokus.

1. Introduction

A large body of evidence supports the role of chronic stress in potentiating the development of numerous diseases. These range from metabolic conditions such as diabetes and obesity to depression, anxiety and other psychiatric disorders. Recent evidence implicates chronic stress as a key risk factor for the onset of cardiovascular disease (CVD) which is of particular concern given the global prevalence of such disorders.

Innate stress-response mechanisms are activated following exposure to harmful stimuli. Over millions of years, such systems evolved to adequately prepare the body against a variety of stressors, i.e. by increasing energy availability and upregulating host defense mechanisms. However, drastic changes accompanying modern-day living conditions mean that most external stressors are neither transient nor physical, thus limiting the capability of the body's inherent stress response to chronic psychosocial stressors.

It is well established that the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) function as the primary effectors of the innate stress response. However, the underlying stress-mediated molecular pathologies driving CVD progression are yet to be fully elucidated. In recent years, the endothelium has emerged as a particularly susceptible target of glucocorticoid and catecholamine action. This review highlights the current burden of stress in South Africa (SA), as well as the role of chronic stress in potentiating CVD onset. It provides a comprehensive overview of the highly integrated stress response before focusing on the potential mechanisms driving vascular dysfunction.

2. Chronic stress: an important component of psychiatric disorders and CVD

Poor mental health constitutes a large portion of the noncommunicable global burden of disease (World Health Organization, 2018). During 2015, mental disorders were directly responsible for 17.3 million years lived with disability and 8.6 million years of life lost, amounting to ~ 232,000 deaths (Sorsdahl *et al.*, 2018). Depression is the most prevalent 12-month and lifetime disorder in SA, with the Western Cape boasting the highest prevalence rates (Quirk *et al.*, 2013; Whiteford *et al.*, 2015). During 2009, the South African Stress and Health study revealed that 1 in 17 Nigerians present with an anxiety or mood disorder, compared to approximately 1 in 6 South Africans (Herman *et al.*, 2009). This correlates with a more recent survey that ranked SA and Nigeria as the most stressed countries in the world, using data sourced from the International Monetary Fund, the United Nations Office on Drugs and Crime, Transparency International, the Central Intelligence Agency World Fact Book and the World Health Organization (Bloomberg, 2013). Stress levels were estimated using the following parameters: income inequality, homicide rates, unemployment, life expectancy, corrupt perception, urban air pollution, gross domestic product per capita and purchasing-power-parity basis (Bloomberg, 2013). Although chronic stress is a key component of poor mental health, it is also highly prevalent in those of the “otherwise-healthy” global population (Ross *et al.*, 2017; Yousaf *et al.*, 2019). This highlights the current burden of poor mental health in SA and why it is essential to gain greater mechanistic insights into the pathological implications of chronic stress.

2.1 Chronic stress and disease

Stress can be defined as the body’s generic response to intrinsic or extrinsic stimuli that threaten to overwhelm its ability to maintain homeostasis (Johnson *et al.*, 1992; McEwen, 2005). Should the stress response be chronically upregulated, damaging and debilitating effects may ensue if excessive and un-checked autonomic and adrenocortical function are not prevented. Although this response elicits broad downstream effects, stressor characteristics can be highly influential.

These include a) the type of stressor (emotional or physical), b) history of early-life stress (ELS) and c) the presence of comorbidities (Johnson *et al.*, 1992; Brosschot, 2010). Emotional stressors continuously challenge homeostasis, emphasizing that adequate physiological and behavioral competence is crucial for maintaining a healthy mental state (Agorastos *et al.*, 2018). Psychological stressors can be further categorized into two domains: environmental and emotional influences. The former refers to low socioeconomic status, family or job stress, traumatic life experiences and low social support. Emotional factors include anxiety, depression and fear (Yammine *et al.*, 2014). Research indicates that the magnitude of the mounted response as well as the ability to recover from various stressors, is dependent on the individual's perception of the stressors rather than the stressor itself. The perception of a stressor(s) is further influenced by a) novelty of the stressor, b) its unpredictability, c) degree of threat to a person or ego and d) the sense of loss of control. Patient characteristics are also profoundly influential and include age, gender and personality traits (Guilliams and Edwards, 2010).

Adverse exposures during neonatal, childhood and adolescent life drastically increase the risk of developing stress-mediated psychiatric disorders (van Bodegom *et al.*, 2017). Here studies demonstrate that the frequency, duration, degree and presence of other contributing risk factors (e.g. drug addiction and poverty) corresponds with disorder complexity and severity (Scott, 2011; Reuben *et al.*, 2016). Approximately 30-40% of the global adult population have experienced ELS, which includes maltreatment, parental separation and abuse (Agorastos *et al.*, 2018). Early-life stress exposure during juvenile brain development is linked to a poorer adaptability to stressors in adult life as well as elevated susceptibility to cardiovascular and neurological diseases (Chrousos, 2009; Scott, 2011). Given large socioeconomic disparities, high poverty rates and poor access to quality healthcare, the SA population is particularly susceptible to the debilitating outcomes of ELS. Due to its extensive public health impact, it was suggested that ELS

be conceptualized as an independent risk factor for various cardiovascular, metabolic and inflammatory conditions (Korkeila *et al.*, 2010; Agorastos *et al.*, 2018).

2.1.1 Chronic stress and CVD

Despite reductions in global incidence rates, CVD persists as the primary source of noncommunicable deaths (World Health Organization, 2018). Aside from the traditional CVD risk factors, recent studies implicate chronic stress as an important driver of CVD, diabetes and metabolic syndrome onset (Lagraauw *et al.*, 2015; Brunner, 2017; Esler, 2017; Sgoifo *et al.*, 2017; Wood and Valentino, 2017). Epidemiological studies such as the INTERHEART study (n = 29,972 participants from 52 countries) and the INTERSTROKE study (n = 26,919 participants from 32 countries) both recognized psychosocial stress as one of ten modifiable risk factors for the onset of heart disease and stroke (Rosengren *et al.*, 2004; O'Donnell *et al.*, 2016). Moreover, behavioral risk factors such as poor nutrition, a sedentary lifestyle as well as hazardous alcohol and tobacco use are all exacerbated by poor mental health (Dimsdale, 2008; Sorsdahl *et al.*, 2018)**.**

Experimental evidence indicates that stressed persons are more likely to consume calorie-rich foods with high levels of sugar, salt and saturated fats (Penninx, 2017). This is concerning as approximately 37% of the adult SA population are physically inactive, with 24% of all adults presenting with raised blood pressure and 27% regarded as clinically obese (World Health Organization, 2014). Within the SA context, data revealed that psychologically distressed individuals exhibited an increased prevalence of hypertension (15.7%) and diabetes (15.0%), both prominent risk factors for CVD onset (Sorsdahl *et al.*, 2018).

Sources of chronic life-stressors are varied and include interpersonal conflict, financial troubles, personal worries and work-related stressors (Wilson *et al.*, 2014). Moreover, a meta-analysis (n = 47,045) found that work stress was associated with poor lifestyle behaviors and diabetes, both of which are important contributors of CVD development (Nyberg *et al.*, 2013; Kivimäki and Kawachi, 2015). Job strain, social isolation and loneliness were also associated with a 50%

increased risk of developing CVD (Steptoe and Kivimäki, 2013; Cohen *et al.*, 2015). A similar pattern holds for the SA population as approximately 36% of persons between the ages of 15 to 29 are not employed, educated or participating in any formal training (Lannoy and Mudiriza, 2019). As this trend has persisted since 2013, it highlights the desperate state a large proportion of South Africans experience and emphasizes the detrimental burden of chronic life-stressors on the SA population.

A number of studies have examined the effects of chronic stress on endothelial function (Table 1). In the context of CVD, healthy endothelium is crucial for the prevention of atherosclerosis and other vascular pathologies (Bonetti *et al.*, 2003). Participants of these studies most commonly suffered from stressors pertaining to occupational challenges and the burden of prolonged care for loved ones with dementia (Table 1). Two distinct methods can be employed to assess *in vivo* human endothelial function. The first method involves measuring fluctuations in blood flow or artery diameter following administration of an endothelium-dependent vasodilator. Flow-mediated dilation (FMD) is an alternative technique used to assess shear-stress induced changes in arterial calibre after a transient period of forearm ischemia (Golbidi *et al.*, 2015). Subsequent to periods of chronic stress, subjects typically present with impaired FMD as well as increased expression of markers of endothelial activation and damage (Table 1).

2.1.2 Depression and CVD

Stress-related psychiatric disorders, such as depression and anxiety, display largely identical neurobiology and thus present with similar symptoms (Hill *et al.*, 2012; Vaccarino and Bremner, 2017). Depression is the leading worldwide cause of disability and is a major contributor to the overall global burden of disease (World Health Organization, 2018). Many studies illustrate the impact of depression on somatic health and CVD risk (Penninx, 2017). A meta-analysis and systematic review integrating longitudinal evidence from 1,608 articles concluded that depression significantly increased the risk of developing CVD, with clinically diagnosed major depressive

disorder being the strongest predictor for CVD morbidity and mortality (Van der Kooy *et al.*, 2007; Doyle *et al.*, 2015). Furthermore, subclinical manifestations of CVD such as atherosclerosis, impaired endothelial function, aortic calcification and arterial stiffness are upregulated in depressed individuals (Penninx, 2017). Another study (n = 52,095) found that depression and post-traumatic stress disorder (PTSD) were independently associated with heart disease (Scott *et al.*, 2013). Depressed individuals who are saddled with CVD are at a higher risk of recurrent cardiovascular complications and mortality (Bartoli *et al.*, 2013; Towfighi *et al.*, 2017). The depression-associated CVD risk could be due to an unhealthy lifestyle, biological dysregulation, early-life trauma, personality traits and genetic vulnerability (Penninx, 2017). It is well documented that depressed individuals consistently consume larger quantities of alcohol and smoke more than non-depressed persons (Penninx, 2017). Additionally, depressed persons are more likely to exhibit higher caloric intake, less frequently engage in physical activity and are more likely to present with certain vitamin deficiencies (Penninx *et al.*, 2011).

Chronic hyperactivation of the HPA axis in clinically depressed individuals is one of the most concrete findings of psychiatric research (Tsigos and Chrousos, 2002). Other biological dysregulations include increased sympathetic tone with subsequent cardiac outcomes (arrythmias, hypertension, increased heart rate variability) (Licht *et al.*, 2013) and metabolic dysregulation (hyperglycemia and hypercholesterolemia) (Vogelzangs *et al.*, 2014). A dysfunctional immune response - characterized by elevated levels of circulating inflammatory mediators - is also associated with depression to further increase CVD risk (Penninx *et al.*, 2011; Liu *et al.*, 2012; Fioranelli *et al.*, 2018).

Table 1: Summary of studies investigating chronic stress and endothelial dysfunction. MESA – Multi-ethnic Study of Atherosclerosis; ICAM-1 - intercellular adhesion molecule-1; FMD – flow-mediated dilation; sTF - soluble tissue factor; VWF - von Willebrand factor.

Study design	Stressor characteristics	Parameters	Findings	Reference
Multi-ethnic sample of adults (45-84 years) from the MESA.	Participants questioned on financial, job, relationship or health-related issues spanning the previous 6 months.	FMD (n = 2,963); ICAM-1 (n = 2,523); E-selectin (n = 971).	Chronic stress associated with lower absolute FMD after adjusting for demographic, socioeconomic, behavioral and biological risk factors. ICAM-1 but not E-selectin was associated with higher stress levels.	(Kershaw *et al.*, 2017)
Multi-ethnic sample of adults (mean age: 56.1 ± 0.21; 55% men; 41.2% white) (n = 1,499) from the MESA.	Stress associated with work-hours, job control, job strain and occupational category.	FMD.	FMD was only positively correlated with work-hours before adjustment for age, gender and CVD risk factors. FMD was associated with occupational category; with management and sales presenting the highest FMD values. No associations	(Charles *et al.*, 2014)

			between FMD and job strain, job control or job demands were observed. Although race had no influence on any correlations, gender modified the association between FMD and occupational stress.	
Elderly caregivers (Mean age = 74.3 ± 8.1; 68% women; 87% white) underwent three yearly assessments.	Stress attributed to prolonged caregiving.	FMD.	Positive association found between leisure satisfaction and FMD. Negative relationship found for stress and FMD. Depressive symptoms were not associated with FMD. Time and number of years of caregiving was a significant predictor of FMD deterioration.	(Mausbach *et al.*, 2012)
Elderly individuals providing care for demented and	Stress as a result of prolonged caregiving.	FMD.	Participants caring for partner with moderate to severe dementia presented with significantly worse FMD than those caring for a spouse with mild dementia	(Mausbach *et al.*, 2010)

nondemented partners (n = 78).			and non-caregivers. Furthermore, the number of years of caregiving was related to severity of FMD deterioration.	
Caucasians (n = 804) from the Survey of Midlife in the United States	Chronic stress stemming from discrimination.	Serological E-selectin.	Women reported a greater number of instances of major and everyday discrimination than men. For men, major and everyday discrimination was associated with higher E-selectin levels.	(Friedman *et al.*, 2009)
PTSD (n = 14) and non-PTSD age- and gender-matched controls (n = 14).	Stress associated with PTSD (re-experiencing, avoidance, arousal).	Three markers of endothelial dysfunction assessed: sTF; VWF; soluble ICAM-1.	Patients with PTSD had higher sTF and VWF levels, with no association found between symptoms of PTSD and soluble ICAM-1.	(von Känel *et al.*, 2008)

2.2 Synopsis

Chronic stress represents an important mental health issue as it is associated with the onset and progression of various diseases. More recently, a large body of evidence supports a role for chronic stress in mediating CVD onset. This is alarming considering the already devastating burden of CVDs on SA's health, well-being and economic productivity (Hofman, 2014). Given the high prevalence of stress amongst South Africans, coupled with poor quality of healthcare, lack of education and extreme poverty, this population is particularly susceptible to the burden of chronic stress and its associated pathologies. This emphasizes the need for the research and development of more effective coping strategies and a push for encouraging the youth to implement these strategies from a younger age.

The next section provides a comprehensive overview of stress-response mechanisms and how they function to preserve homeostasis. Maladaptions of these systems following exposure to chronic stress will also be reviewed.

3. The major stress-response mechanisms

3.1 Brief history of stress research

Although the concept of maintaining a stable internal environment dates back to Greek philosophers such as Empedocles and Hippocrates (Garrison, 1929; Adams, 1939), it was Walter Cannon who coined the term “homeostasis” in the early 1900s. He demonstrated that emotional and physical stimulants induced a similar response and that this was partly facilitated by the sympathoadrenal axis (Cannon, 1929). Almost 30 years later, the “General Adaptation Syndrome” theory was put forth by Hans Selye (Selye, 1936; Szabo *et al.*, 2012). With this theory, Selye pioneered the field of stress biology and highlighted the importance of the HPA axis in maintaining this stable internal milieu (Szabo *et al.*, 2012). In 1948, a former student of Selye proposed that the function of the anterior pituitary gland was modulated by factors released from the hypothalamus (Harris, 1948). This factor was then named corticotropic releasing hormone (CRH) in 1955 (Saffran, Schally and Benfey, 1955). Current research is directed towards elucidating the downstream negative effects of a chronically upregulated stress response and how such maladaptions can lead to pathophysiologic outcomes and disease onset.

3.2 The integrated stress response

Appropriate basal activity of the stress system is important for a sense of well-being, efficient performance of tasks and positive social engagements (Chrousos, 2009). Inadequate responsiveness of the stress response (in terms of both duration and magnitude) may impair growth and development and also contribute to endocrine, behavioral, cardiovascular, metabolic and immune disorders (Yousaf *et al.*, 2019). The stress-mediated progression of such conditions is reliant upon the genetic vulnerability or resilience of an individual to stress, their exposure to traumatic events during juvenile development as well as the severity, duration and timing of the stressor (Chrousos, 2009; Terenina *et al.*, 2019).

Allostasis can be defined as the process of maintaining homeostatic stability with the help of various physiological regulatory systems that regulate the release of hormones and other mediators (Seeman *et al.*, 2001). The perpetual upregulation of this response results in physiological wear and tear, particularly following impaired negative feedback inhibition. This concept is known as allostatic load and can lead to alterations in various neuroendocrine set-points. Although such adaptions confer immediate benefits, they can also elicit deleterious effects in the long-run (McEwen, 1998, 2005). Sustained allostatic overload therefore results in a maladaptive stress response, psychological disturbances and metabolic dysregulation (Golbidi *et al.*, 2015). Although the links between poor mental health and CVDs are yet to be elucidated, upregulation of the HPA axis and SNS is paramount. The following sections review the activity of these systems following exposure to acute and chronic stressors.

3.2.1 HPA axis

Acute response

Activation of the HPA axis begins with the release of CRH from hypophysiotropic neurons of the hypothalamus (Johnson *et al.*, 1992; Burford *et al.*, 2017). This hormone is then transported to the anterior pituitary gland via hypophyseal portal vessels where they stimulate the release of ACTH (Figure 1). This hormone then binds to melanocortin type 2 receptors in the adrenal cortex which in turn triggers the release of cortisol (corticosterone in animals) from the zona fasciculata and the zona reticularis (Charmandari *et al.*, 2005). Glucocorticoids largely exert metabolic effects that mobilize biochemical resources to aid in the “fight-or-flight” response. Aside from its hyperglycemic affects, cortisol also acts on the hypothalamus and anterior pituitary as part of a negative feedback mechanism to inhibit the secretion of CRH and ACTH (Figure 1) (Burford *et al.*, 2017).

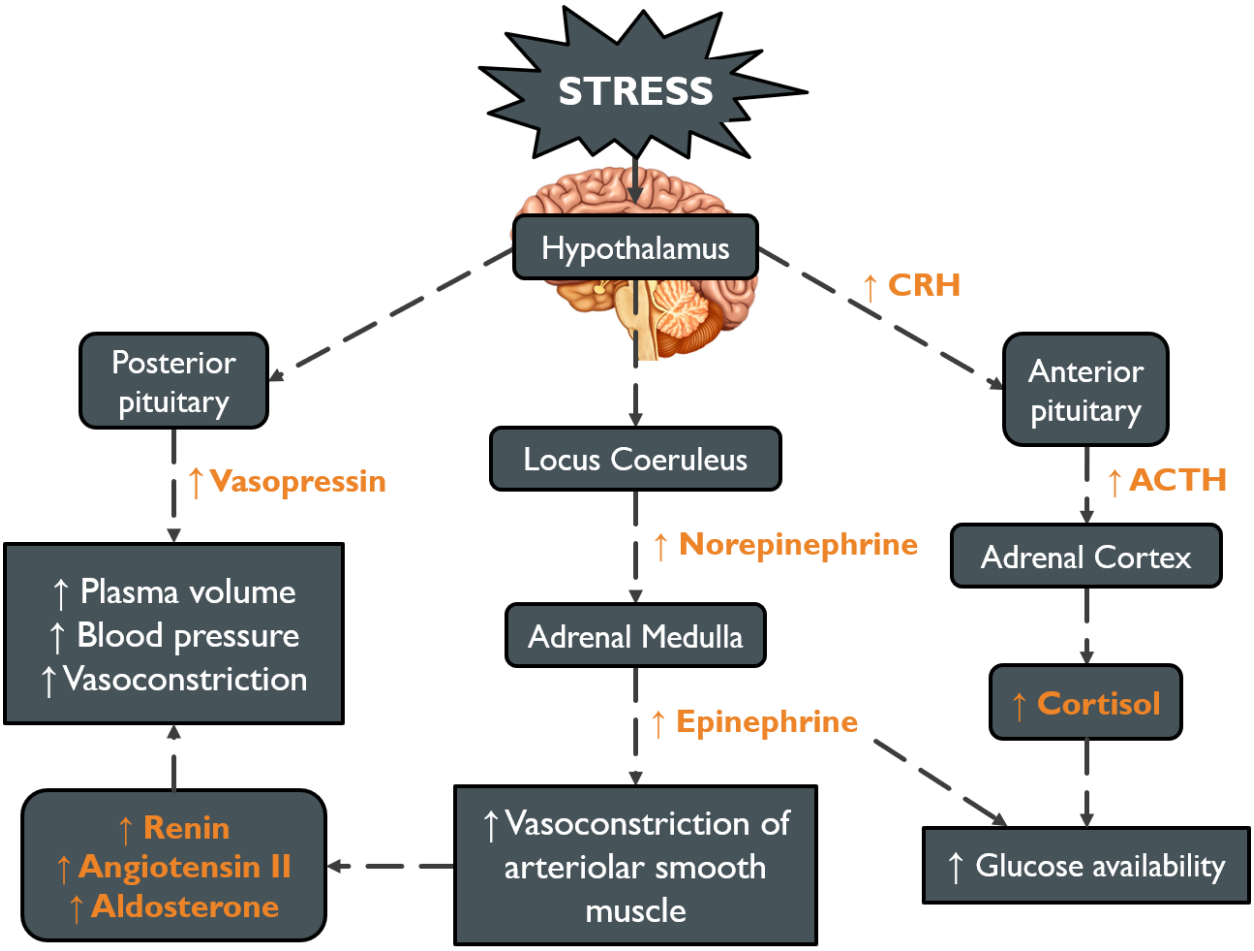

Figure 1: The integrated stress response. CRH – Corticotropic-releasing hormone; ACTH – Adrenocorticotropic hormone.

Corticotropic-releasing hormone is a 41 amino-acid peptide and was isolated and characterized by Vale in 1981 (Vale *et al.*, 1981). This peptide hormone is produced in the neuronal cell bodies of the paraventricular nucleus. Corticotropic-releasing hormone receptors are not only found in the hypothalamus but are also present in the limbic system, the basal forebrain and locus coeruleus-norepinephrine (LC-NE) system of the brainstem; indicating the broad regulatory role of this peptide in the stress response (Charmandari *et al.*, 2005). Corticotropic-releasing hormone binds to two receptors, namely CRH receptor-1 and CRH receptor-2 (CRHR1 and CRHR2) (Deussing and Chen, 2018). Although both are G protein-coupled receptors, CRHR1 is predominantly found in the central nervous system whereas CRHR2 is localized peripherally

(vasculature, heart and gastrointestinal tract) (Stenzel *et al.*, 1995; Burford *et al.*, 2017). Various stimuli regulate CRH production and secretion, such as fear or anxiety, fluctuations in blood pressure and pain. Emotions influence CRH secretion as a result of neural connections between the paraventricular nucleus and limbic system. Other regulatory factors include catecholamines, acetylcholine, serotonin and inflammatory interleukins (ILs) (Deussing and Chen, 2018). Studies on mice deficient in both CRH receptors demonstrate immune, reproductive- and cardiovascular-modulatory effects (Jeong *et al.*, 1999). Direct effects of CRH on the cardiovascular system include elevated heart rate, increased cardiac output as well as elevated mean arterial pressure via the induction of NE and epinephrine (E) release (Deussing and Chen, 2018).

Proopiomelanocortin is the precursor for ACTH and is synthesized in multiple brain sites, such as the arcuate nucleus of the hypothalamus, the LC and the pituitary gland. Proopiomelanocortin is cleaved in the anterior pituitary gland to produce ACTH (39 amino-acid peptide) and β-endorphin (92 amino-acid fragment) (Harno *et al.*, 2018). Systemic circulation transports ACTH to the adrenal gland where it exerts a trophic effect as well as promoting the production and secretion of glucocorticoids, aldosterone and adrenal androgens (Figure 1) (Miller and Auchus, 2011). This means that regular exposure of adrenal tissue to fixed doses of ACTH, induces adrenal cortex hypertrophy and progressively increases the amount of glucocorticoids that are secreted – as evident in chronic stress (Bernatova *et al.*, 2018). Furthermore, ACTH receptors are desensitized and downregulated when ACTH concentrations are high, and *vice versa* (McCarty, 2016). Although CRH is the most potent stimulator of ACTH secretion, other stimulants include arginine vasopressin, oxytocin, angiotensin II, serotonin, NE and E (Harno *et al.*, 2018).

Glucocorticoids play a key role in stress adaption by increasing the availability of biochemical resources as well as facilitating memory and learning (Table 2) (Myers *et al.*, 2017). Due to the lipophilic nature of cortisol, its rate of secretion is controlled by regulation of its synthesis. Secreted cortisol is largely bound to transcortin and is inactive in this form. Approximately 5% of the free

circulating cortisol represents the active fraction which is in turn responsible for negative feedback inhibition on CRH and ACTH production (Johnson *et al.*, 1992). Two types of glucocorticoid receptors exist and mediate this hormone's effects. Type I receptors are predominantly found throughout structures that comprise the limbic system. Due to this system's involvement in fear and anxiety, stimulation of such receptors alters behavioral responses to environmental and emotional stimuli, with subsequent changes in HPA axis activity (Burford *et al.*, 2017). These receptors also display a high affinity for cortisol and are similar in structure to mineralocorticoid receptors. Type 2 glucocorticoid receptors are predominantly found in CRH neurons of the hypothalamus. Other important sites include the amygdala, hippocampus and nucleus tractus solitarius (Johnson *et al.*, 1992), demonstrating that such receptors participate in behavioral, neuroendocrine and autonomic responses to stress.

Glucocorticoids exert their effects by binding to specific receptors found in the cytoplasm of target cells. The hormone-receptor complex subsequently migrates to the nucleus, where it binds to the complementary hormone-response element found within the target DNA (deoxyribonucleic acid). This initiates the transcription of specific genes involved in metabolism, immune function, growth, inflammation and cognition (Oakley and Cidlowski, 2015). The primary metabolic outcome of glucocorticoids is the induction of hyperglycemia at the expense of protein and fat stores (Figure 1). This is achieved by the upregulation of gluconeogenesis, glycogenolysis, protein degradation and lipolysis (Tsigos and Chrousos, 2002). Stress is often accompanied by tissue injury and an exaggerated inflammatory response. As this has the potential to cause harm, cortisol interferes with almost every step of the inflammatory pathway (Table 2). For example, glucocorticoids attenuate neutrophil phagocytosis, inhibit the production of inflammatory mediators and reduce antibody production by lymphocytes (Burford *et al.*, 2017).

Maladaptions in HPA axis activity following chronic stress exposure

Evidence suggests that sustained hypo- or hyperactivation of the HPA axis is dependent upon the individuals' age of exposure to and the duration of various stressors (van Bodegom *et al.*, 2017). However, most models of HPA axis dysregulation follow Selye's General Adaptation Syndrome theory, suggesting that chronic stress elicits an eventual shift from a hyper- to a hypo-responsive HPA axis (Selye, 1936). Initially, excessive hypothalamic and pituitary stimulation leads to increased glucocorticoid and decreased dehydroepiandrosterone production (Burford *et al.*, 2017). Dehydroepiandrosterone is a glucocorticoid antagonist that is produced by the adrenal cortex and exerts beneficial effects, including the suppression of systemic inflammation and the attenuation of neurologic damage as a result of dysregulated cortisol secretion (Walker *et al.*, 2017). Although concurrent increases in glucocorticoid receptor (GR) sensitivity and expression occur, the number of GRs eventually subside. This leads to glucocorticoid resistance and impaired negative feedback inhibition of the HPA axis (Table 2) (Merkulov *et al.*, 2017). Despite the inherent anti-inflammatory influence of glucocorticoids, this hormone also binds to mineralocorticoid receptors to subsequently induce a proinflammatory response (Hannibal and Bishop, 2014). Under normal conditions, this effect is prevented by enzymes that block the binding of cortisol with mineralocorticoid receptors. Reduced GR expression is further paralleled with adrenal cortex fatigue which in turn leads to hypocortisolism. This adaption is thought to be protective as it prevents chronic, glucocorticoid-induced immunosuppression and metabolic catalysis. Downregulation of CRH receptors further promotes hypocortisolism (Guilliams and Edwards, 2010).

Table 2: Effects of chronic stress-induced hyper- and hypocortisolism.

Effects of hypercortisolism	Effects of hypocortisolism
Generalized immunosuppression (↓ leukocyte traffic and function, ↓ production of cytokines and blunted effects of inflammatory mediators on target tissue) (Burford *et al.*, 2017).	Immune activation (↑ production of proinflammatory cytokines, such as tumour necrosis factor-α, IL-6 and IL-12) and ↓ production of anti-inflammatory cytokines (IL-4 and IL-10) (Elenkov and Chrousos, 1999).
Inhibited reproductive axis (↓ gonadotropin-releasing hormone) (Guilliams and Edwards, 2010).	Hypotension, hypoglycemia, weight loss and enhanced fatigability (Guilliams and Edwards, 2010).
Upregulated catabolism (↑ visceral fat deposition, ↓ lean body mass and ↓ osteoblast activity) (Siti *et al.*, 2015).	State of hypoarousal (Charmandari *et al.*, 2005).
Inhibition of growth axis (↓ secretion of growth factors, such as growth hormone and insulin-like growth factor-1, and ↑ somatostatin production) (Charmandari *et al.*, 2005).	

3.2.2 The SNS

Acute response

In 1929, Walter Cannon first described the activation of the LC-NE system following exposure to various stimuli (Cannon, 1929). This system is largely contained within the brainstem and is highly integrated with and regulated by the hypothalamus, the cerebral cortex and the limbic system. Following activation, catecholamines are released into systemic circulation by sympathetic neurons and the adrenal medulla. These hormones induce various physiological adaptions, such as increased heart rate and cardiac output, simultaneous vasoconstriction and vasodilation as well as increased energy availability. Cognitive outcomes include a heightened sense of arousal and increased anxiety (Myers *et al.*, 2017).

Norepinephrine and E exert their effects via second messenger pathways and exhibit differing affinities for four distinctive adrenergic receptors, namely α_1, α_2, β_1 and β_2. Norepinephrine and adrenomedullary E bind to α_1 and β_1 receptors located near terminal sites of postganglionic sympathetic neurons (Kvetnansky *et al.*, 2009). Thus, NE and E exert similar effects on target tissues, with E reinforcing SNS activity. However, E also possesses a high affinity for β_2 receptors, which the SNS has little influence over. This is partially because β_2 receptors are located in select tissues like skeletal muscle and bronchiolar smooth muscle. Epinephrine selectively promotes vasodilation of vessels supplying the heart and skeletal muscle, thereby increasing blood flow to these areas (Dyson *et al.*, 2006; Golbidi *et al.*, 2015). Arterioles in the digestive tract and the kidneys are only equipped with α_1 receptors and therefore undergo more profound vasoconstriction during SNS activity (Charmandari *et al.*, 2005).

Maladaptions in SNS activity following chronic stress exposure

Chronically stressed animals typically present with a hypersensitive SNS. This leads to hypertrophy and hyperplasia of adrenal tissue, with the concurrent upregulation of enzymes responsible for catecholamine synthesis (Johnson *et al.*, 1992). Interestingly, exposure to familiar

stressors leads to a reduced sympathomedullary response. Contrastingly, novel stressors induce exaggerated catecholamine release from the adrenal medulla (Groeschel and Braam, 2011; Golbidi *et al.*, 2015). Autonomic-induced sustained upregulation of the renin-angiotensin-aldosterone system (RAAS) system further leads to hypertension, vascular damage as well as a proinflammatory and prooxidative milieu (Häfner *et al.*, 2012).

Thus, the integrated stress response is highly complex and employs a wide variety of effectors to restore homeostasis by mobilizing biochemical resources and upregulating host defense mechanisms. However, most modern-day stressors present in the form of chronic existential, financial, family or personal worries. Such stressors lead to the sustained upregulation of the stress mechanisms which eventually result in alterations of physiological set-points as well as the "wear-and-tear" of various systems and structures. Although this may manifest in a multitude of different ways, the vasculature emerges as a crucial target as it is susceptible to excessive systemic levels of catecholamines and glucocorticoids.

The next section highlights and explains the deleterious effects of stress hormones on the vasculature, with emphasis placed on how endothelial dysfunction may arise.

4. Chronic stress and endothelial dysfunction: proposed mechanisms

Endothelial cells line the inner wall of all vasculature and play an essential role in maintaining vascular homeostasis (Incalza *et al.*, 2018). The endothelium releases factors (e.g. paracrines) in response to chemical or physical challenges which function to locally regulate arteriolar calibre. For example, endothelial nitric oxide synthase (eNOS) produces nitric oxide (NO) which induces vasodilation by increasing cyclic guanosine monophosphate levels in smooth muscle cells (Fleming and Busse, 2003; Searles, 2006). A large body of evidence demonstrates that endothelial dysfunction plays a critical role in the pathogenesis of stress-mediated atherosclerosis and CVD (Lind *et al.*, 2002; Spieker *et al.*, 2002; Golbidi *et al.*, 2015). This is characterized by endothelial activation, the release of adhesion molecules, impaired vascular reactivity as well as a proinflammatory, prooxidative and prothrombotic state (Figure 2) (Higashi *et al.*, 2014).

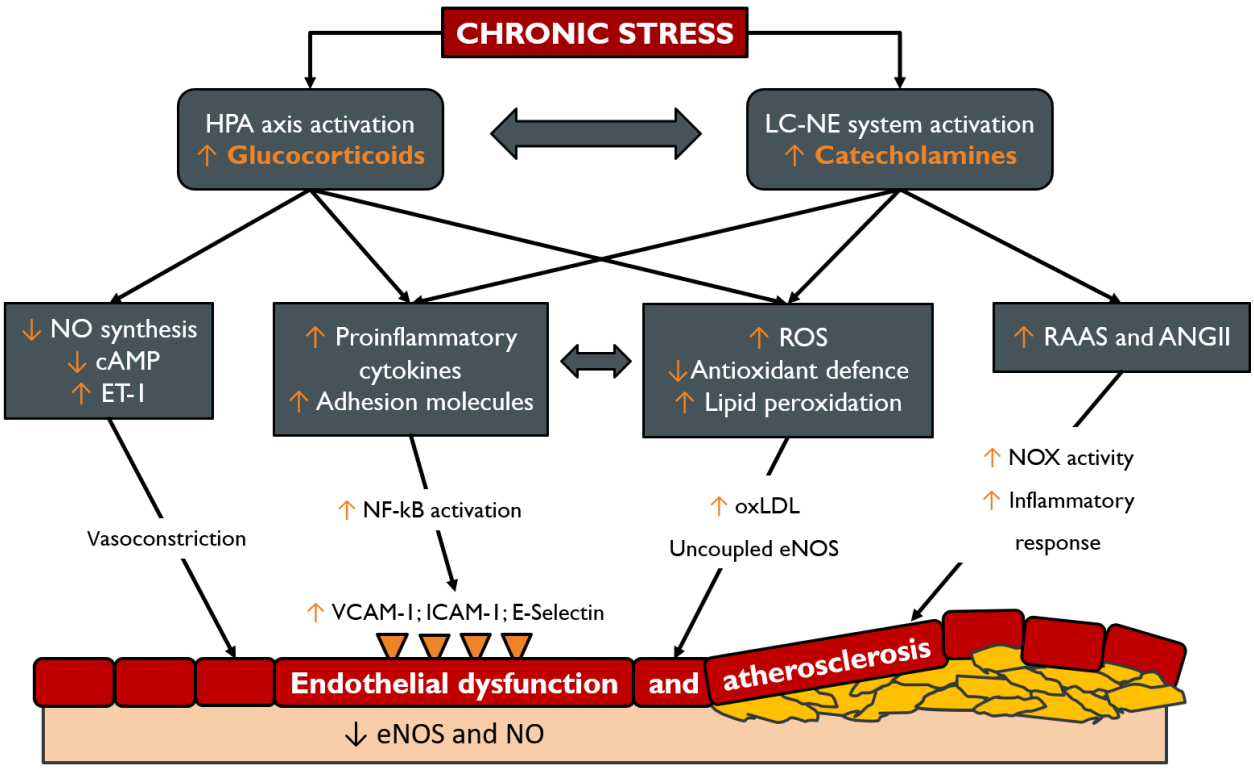

Figure 2: Potential mechanisms of chronic stress-induced endothelial dysfunction. HPA – Hypothalamic-pituitary-adrenal; NO – nitric oxide; cAMP – cyclic adenosine monophosphate; ET-

1 – endothelin-1; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells; VCAM-1 - vascular cell adhesion molecule-1; ICAM-1 – intercellular adhesion molecule-1; eNOS – endothelial nitric oxide synthase; LC-NE – locus coeruleus-norepinephrine; ROS – reactive oxygen species; oxLDL – oxidized LDL; RAAS – renin-angiotensin-aldosterone system; ANGII – angiotensin II; NOX – NADPH oxidase.

Atherosclerosis is the primary underlying pathology of CVD, with chronic stress also identified as a key driver of atherogenesis (Yao *et al.*, 2019). This condition is characterized by the formation of atherosclerotic plaques in the walls of medium- to large-sized arteries (Ross, 1993; Bäck *et al.*, 2019). These lesions ensue following retention and oxidation of low-density lipoproteins (LDL), that is exacerbated by the presence of a chronic, low-grade inflammatory state. Maintaining endothelial integrity is crucial for preventing plaque development as lesions first occur in sites of dysfunctional and denuded endothelium (Mudau *et al.*, 2012). Cholesterol is packaged in LDL which in turn infiltrates the vascular subendothelial space through defective and leaky endothelial cells. Here proatherogenic lipoproteins are oxidized and subsequently become proinflammatory and cytotoxic. With the help of cytokines (IL-8) and chemokines (monocyte chemotactic protein-1), monocytes migrate across the dysfunctional endothelial layer. Once within the intima, newly formed macrophages engulf lipoproteins and become foam cells. Such cells continue to phagocytose LDL molecules until they die either via apoptosis or necrosis. The accumulation of dead, lipid-rich macrophages within the intima eventually results in the formation of a plaque, characterized by a destabilizing core and a fibrous cap (Falk, 2006). Inflammatory and hemodynamic forces degrade the cap and expose prothrombotic content into the blood. This triggers the clotting cascade, leading to vessel occlusion, myocardial infarction and stroke (Kasikara *et al.*, 2018; Bäck *et al.*, 2019).

The following sections discuss the role glucocorticoids and catecholamines play in driving endothelial dysfunction and atherogenesis.

4.1 Glucocorticoids and endothelial dysfunction

A large body of evidence supports a role for glucocorticoid-mediated endothelial dysfunction. Sustained circulating cortisol levels may lead to a variety of downstream vasculopathies by a) reducing endothelial NO synthesis, b) decreasing cyclic-adenosine monophosphate (cAMP) levels, c) interacting with GRs and d) promoting the secretion of endothelin-1 (ET-1) (Figure 2) (Nickel *et al.*, 2009; Yammine *et al.*, 2014; Golbidi *et al.*, 2015).

Reduced NO bioavailability is one of the hallmark features of endothelial dysfunction and is associated with atherosclerotic plaque development, as well as a proinflammatory and prothombotic state (Bonetti *et al.*, 2003; Kalanuria *et al.*, 2012). Endothelial NOS depends on the catalytic capabilities of five cofactors to effectively synthesize NO, i.e. flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin, heme and calcium/calmodulin (Endemann, 2004). If any cofactors are absent, or if there is limited availability of the substrate L-arginine, eNOS produces superoxide (SO) instead of NO. Superoxide is a potent free radical and its deleterious effects will be discussed in more detail later (Malekmohammad *et al.*, 2019). Glucocorticoids directly reduce NO availability by inhibiting eNOS activity and expression (Figure 2) (Liu *et al.*, 2009; Toda and Nakanishi-Toda, 2011). In support, a study investigating the effects of cortisol on human umbilical endothelial cells showed that it lowered NO synthesis by 60% while GR antagonists ameliorated this effect (Wallerath *et al.*, 1999).

Adequate cAMP is crucial for intracellular signal transduction as well as the production and maintenance of endothelial cells (Fukuhra *et al.*, 2006). Cyclic AMP also exerts important anti-inflammatory properties via its actions on nuclear factor kappa-light-enhancer of activated B cells (NF-κB) kinase and tumour necrosis factor-α (TNF- α) (Golbidi *et al.*, 2015). Elevated endothelial cAMP can reduce hyperglycemia-induced oxidative stress by attenuating reactive oxygen species (ROS) production. This is important as high blood glucose levels are often comorbid with patients

suffering from poor mental health and CVD. An inverse relationship between oxidized LDL, atherosclerotic plaque development and cAMP also exists (Fantidis, 2010).

Glucocorticoid receptors directly influence endothelial cells by modulating the expression of proinflammatory cytokines and chemokines (IL-6, IL-8 and monocyte chemotactic protein-1), cellular adhesion molecules (ICAM-1, vascular cell adhesion molecule-1 and E-selectin), vasodilators (NO) and vasoconstrictors (angiotensin II and ET-1) which are all crucially involved in preserving endothelial integrity and function (Zielińska *et al.*, 2016; Burford *et al.*, 2017). These receptors also influence blood pressure by sensitizing catecholamines and other vasoactivators. Additionally, GR-specific knockout mice display increased eNOS and inducible NOS expression (Liu *et al.*, 2009).

Recent studies implicated ET-1 as an important mediator of CVD development following chronic upregulation of the acute stress response (Yammine *et al.*, 2014). ET-1 binds with the endothelin-A receptor and contributes to the hypertensive response following chronic stress exposure (Fox *et al.*, 2018). Both experimental and population-based studies demonstrated increased ET-1 levels following psychosocial stress exposure in healthy and diseased subjects (Hong *et al.*, 2006; Fernandez *et al.*, 2010; Wilbert-Lampen *et al.*, 2010; Burg *et al.*, 2011; Fox *et al.*, 2018).

4.2 Catecholamines and endothelial dysfunction

A surge of catecholamines are released following upregulation of the SNS. Norepinephrine and E negatively impact the endothelium by a) directly binding to adrenergic receptors, b) upregulating the RAAS and c) eliciting a prothrombotic state (Figure 2).

Evidence supporting the direct effects of catecholamines on endothelial function is controversial. These hormones exert pressor effects on vasculature which directly oppose that of endothelial-derived NO (Golbidi *et al.*, 2015). Some showed that the administration of exogenous NE increased contractile responses (Iveta *et al.*, 2012), while others demonstrated the protective

effects of β-receptor antagonists by preventing endothelial dysfunction and atherosclerotic plaque development (Boyle *et al.*, 2005). Similarly, α-blockers attenuate SNS-induced reductions in FMD. However, experimental infusions of NE failed to replicate reductions in FMD as observed in human subjects following exposure to mental stressors (Spieker *et al.*, 2002). Acute stress can also promote eNOS activity (Seta *et al.*, 2001). Conflicting findings may be a consequence of differing experimental protocols used for the upregulation of the SNS in human subjects. An important study in this regard investigated the four primary methods of SNS stimulation (cold pressor test, lower body suction, activation of the muscle chemoreflex and the mental arithmetic task) and found that reductions in FMD were not common amongst all tests (Dyson *et al.*, 2006). Thus, it seems more likely that the indirect effects of catecholamines are responsible for inducing endothelial dysfunction.

Chronically stressed and depressed individuals present with a perpetually upregulated RAAS (Figure 2) (Groeschel and Braam, 2011; Häfner *et al.*, 2012). β-blockers such as propranolol can prevent increased RAAS activity (Clamage *et al.*, 1976), while symptoms of anhedonia are ameliorated following the administration of angiotensin-converting enzyme inhibitors (Martin *et al.*, 1990; Okuyama *et al.*, 1999). Angiotensin II (ANGII) is a potent vasoconstrictor and a primary effector of the RAAS. Furthermore, this hormone facilitates atherogenesis by promoting lipid peroxidation (Keidar *et al.*, 1995), hindering cholesterol efflux out of macrophages (Kaplan *et al.*, 2002) and stimulating the uptake of oxidized LDL by macrophages (Keidar *et al.*, 2001).

The binding of ANGII to type-1 receptors enhances the translocation and adhesion of leukocytes (Mateo *et al.*, 2006, 2007) and augments the secretion of proinflammatory cytokines by endothelial and vascular smooth muscle cells (Liu *et al.*, 2006; Chan and Leung, 2007). Candesartan is an angiotensin receptor antagonist that can confer cardiovascular benefits as well as improve HPA axis and SNS-associated responses to psychological stress (Pavel *et al.*, 2008). Furthermore, obliteration of the ANGII type-1 receptor gene ameliorates the progression of plaque

development in apolipoprotein E-deficient mice (Wassmann *et al.*, 2004) and reduces the number of macrophages found in plaques (Fukuda *et al.*, 2008). Studies on ANGII type-2 receptors indicate that they may provide cardioprotective benefits by downregulating SNS activity (Grobe *et al.*, 2007; Lima *et al.*, 2013).

4.3 Inflammation, oxidative stress and endothelial dysfunction

Experimental studies report that chronically stressed humans and animals consistently present with a sustained, low-grade inflammatory and prooxidative state (Howren *et al.*, 2009; Dowlati *et al.*, 2010; Hiles *et al.*, 2012; Siti *et al.*, 2015) This state is characterized by an increased expression of proinflammatory mediators such as IL-6, IL-1β, C-reactive protein and TNF-α. In fact, evidence suggests bidirectional feedback between inflammation and depression as poor adherence to regular levels of physical activity or anti-inflammatory medications reversibly facilitates depression onset (Cohen *et al.*, 2015). Inappropriate immune activation and excess ROS play a considerable role in propagating endothelial dysfunction (Figure 2). Inflammatory mediators may induce vascular pathology by a) decreasing the expression of eNOS mRNA (messenger ribonucleic acid) (Zhang *et al*., 1997), b) modulating calcium channel activity and expression (Tiwari *et al.*, 2006), c) increasing ROS expression (Davignon, 2004) and d) enhancing cyclooxygenase expression (Mitchell *et al.*, 1995).

Immune reactions lead to the production of oxygen radicals as they play a crucial role in repair and defense mechanisms. In a similar way, ROS attracts immune cells and further exacerbates inflammatory reactions (Tang *et al.*, 2015). Oxidative stress activates the NF-κB pathway which mediates inflammatory responses, regulates DNA transcription, activates the endothelium and influences cell growth (Baldwin Jr, 1996; Barnes and Karin, 1997). It was proposed that NF-κB may be the link between chronic stress- and oxidative stress-induced organ dysfunction and impaired vascular regulation (Bierhaus *et al.*, 2004). Inactive NF-κB is primarily found in the cytoplasm and bound to IκB (IKK) proteins. The activation of IKK-α and IKK-β leading to the

phosphorylation and degradation of IκB. The now liberated NF-κB migrates to the nucleus where it promotes proinflammatory cytokine expression, such as ILs, TNF-α, lipoxygenase, cyclooxygenase-2, adhesion molecules and inducible NOS (Golbidi *et al.*, 2015). Furthermore, the anti-inflammatory effects of glucocorticoids on the endothelium are mediated by GR repression of NF-κB (Goodwin *et al.*, 2013).

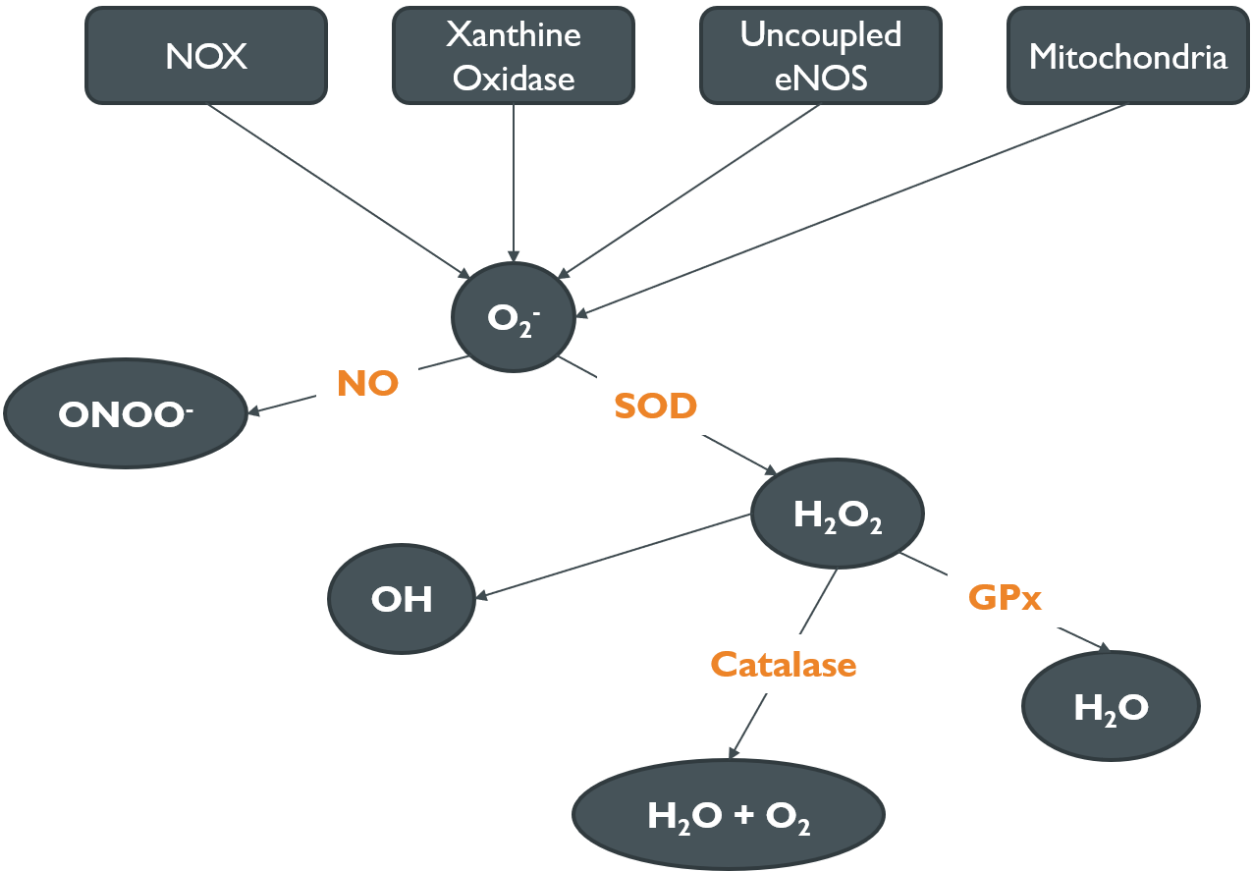

Figure 3: The important role of SO and SOD in the vasculature. NADPH oxidase (NOX), xanthine oxidase, uncoupled eNOS and the mitochondrion are all important sources of SO (O_2^-) in the vascular wall. SO can react with nitric oxide (NO) to form toxic peroxynitrite ($ONOO^-$). SOD is responsible for converting SO into hydrogen peroxide (H_2O_2). H_2O_2 can then spontaneously form a hydroxyl radical (OH) or be converted into H_2O and oxygen (O_2) by catalase or glutathione peroxidase (GPx), respectively.

Oxygen radicals aid in host defense mechanisms, facilitate bacterial destruction and act as second messengers to help orchestrate intracellular signals (Incalza *et al.*, 2018). Oxidative stress occurs when the production of ROS exceeds the buffering capabilities of antioxidants defenses (Figure 3) (Malekmohammad *et al.*, 2019). There are many sources of ROS in the vasculature, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, perturbed eNOS functionality, mitochondrial enzymes and xanthine oxidase (Figure 3). Contrastingly, antioxidant defenses counteracting the excess ROS include superoxide dismutase (SOD), glutathione peroxidase and catalase (Figure 3) (Higashi *et al.*, 2014; Santilli *et al.*, 2015).

Oxidative stress is thought to contribute to cardiovascular pathologies and augment atherosclerotic plaque development by inducing endothelial dysfunction, promoting lipid peroxidation, inflammation and insulin resistance (Pennathur and Heinecke, 2007; Santilli *et al.*, 2015). Cortisol promotes ROS formation via its effects on NADPH oxidase (NOX) and SOD. Furthermore, ROS activate the phosphoinositide 3-kinase pathway which ultimately results in the inhibition of eNOS expression and activity and further reductions in NO synthesis (Virdis *et al.*, 2013; Higashi *et al.*, 2014; Incalza *et al.*, 2018).

Nicotinamide adenine dinucleotide phosphate oxidases are a family of prooxidative enzymes that are characterized by a membrane-bound subunit (Virdis *et al.*, 2013; Santilli *et al.*, 2015; Liao *et al.*, 2018). Angiotensin II is a key activator of NOX (Higashi *et al.*, 2014), with the latter localized in vessel walls and a key source of SO production (Santilli *et al.*, 2015). Other sources of SO include the mitochondrial electron transport chain, uncoupled eNOS and xanthine oxidase (Figure 3). Superoxide can contribute to the formation of other ROS such as the hydroxyl radical, hydrogen peroxide and peroxynitrite (Incalza *et al.*, 2018). High ROS levels lead to the oxidation of the eNOS cofactor, tetrahydrobiopterin and results in the uncoupling of eNOS which further promotes SO instead of NO production. Tetrahydrobiopterin was subsequently identified as an important biomarker for measuring endothelium-dependent vasodilation and SO production

(Malekmohammad *et al.*, 2019). Additionally, SO combines with NO to form toxic peroxynitrite which facilitates protein nitration and endothelial cell death (Figure 3) (Siti *et al.*, 2015).

5. Conclusion

Chronic stress is not only a key element of psychiatric disorders, but in recent years emerged as an underestimated risk factor and potentiator of CVDs. The associative link between chronic stress and CVD was firmly established by a variety of large-scale epidemiological studies (Rosengren *et al.*, 2004; O'Donnell *et al.*, 2016; Sgoifo *et al.*, 2017). Although the underlying molecular mechanisms driving CVD onset are largely unknown, experimental research demonstrates that the upregulation of the HPA axis and SNS are of paramount importance (Johnson *et al.*, 1992; Chrousos, 2009). Glucocorticoids and catecholamines represent the primary effectors of such systems and exert a vast array of downstream cardiac, metabolic and behavioral effects. A healthy endothelial layer is crucial for maintaining cardiovascular and hematological homeostasis. However, this single layer of cells is susceptible to the deleterious effects of excessive, systemic glucocorticoid and catecholamine levels. These hormones may directly decrease NO synthesis, perpetually upregulate the RAAS and promote the secretion of potent vasoconstrictors. Furthermore, they can indirectly facilitate endothelial damage and dysfunction by encouraging a proinflammatory, prooxidative and prothrombotic milieu (Figure 2) (Golbidi *et al.*, 2015; Incalza *et al.*, 2018; Yao *et al.*, 2019; Yousaf *et al.*, 2019). Inflammatory mediators and toxic free radicals further exacerbate this response and augment eNOS uncoupling, lipid peroxidation and ultimately promote atherogenesis (Figure 2). Should this dysfunction go untreated, it may lead to impaired vascular compliance, the development of atherosclerotic lesions, hypertension and blunted tissue function.

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Chapter 2:

Two Months of Unpredictable Chronic Mild Stress Impairs Endothelial Function in Rats

Abstract

Chronic stress plays an increasingly integral part of modern-day living. Evidence suggests that inadequate stress-coping mechanisms facilitate the development of cardiovascular disease. Endothelial dysfunction is characterized by the reduced bioavailability of nitric oxide and represents an important step in disease progression. To investigate whether non-habituating stress impairs endothelial function, we subjected male Wistar rats (weighing 200 – 250 g) to eight weeks of unpredictable chronic mild stress (UCMS). After euthanasia, we measured plasma concentrations of stress-hormones, assessed aortic vascular reactivity to vasodilators and vasoconstrictors as well as investigated the activity of antioxidant enzymes. Eight weeks of UCMS had no effect on circulating corticosterone or epinephrine levels, however plasma adrenocorticotropic hormone was significantly reduced. We noted blunted acetylcholine-induced vasorelaxation with no differences in phenylephrine-induced vasoconstriction. Additionally, we observed reduced vascular superoxide dismutase activity. Collectively, our data supports a role for chronic stress-induced impairments in endothelial-dependent dilation. This may due to increased oxidative stress levels, uncoupled endothelial nitric oxide synthase and reduced nitric oxide availability.

Opsomming

Chroniese stres speel toenemend ’n integrale rol in die hedendaagse lewe. Bewyse dui daarop dat onvoldoende meganismes vir streshantering die ontwikkeling van kardiovaskulêre siektes bevorder. Endoteel wanfunksie word deur die verminderde biobeskikbaarheid van stikstofoksied gekenmerk en is 'n aanduidende stap in die progressie van siektes. Om te ondersoek of nie-gewoontevormende stres ten einde die endotheelfunksie belemmer, het ons manlike Wistar-rotte (wat 200 - 250 g weeg) aan onvoorspelbare chroniese ligte spanning (UCMS) vir ’n tydperk van agt weke onderwerp. Na genadedood het ons plasmakonsentrasies van streshormone gemeet asook die aorta-vaskulêre reaktiwiteit teenoor vasodilators en vasokonstriktore beoordeel en die werking van antioksidantensieme ondersoek. Agt weke UCMS het geen effek op die sirkulerende kortikosteroon- of epinefrienvlakke getoon nie. Die adrenokortikotropiese hormoon van plasma was wel aansienlik verlaag. Ons het kennis geneem van afstomping van asetielcholien-geïnduseerde vaso-ontspanning sonder verskille in fenielefrien-geïnduseerde vasokonstriksie. Verder het ons 'n verminderde vaskulêre superoksied-dismutase-aktiwiteit waargeneem Gesamentlik ondersteun ons data 'n rol vir chroniese stres-geïnduseerde inkortings in endotheel-afhanklike dilatasie. Dit kan wees as gevolg van verhoogde oksidatiewe stresvlakke, ongekoppelde endoteel stikstofoksiedsintase en verminderde beskikbaarheid van stikstofoksied.

1. Introduction

As the developing world continues to expand, stress plays an increasingly central role in the modern-day way of life. Although transient stress exposure is essential for healthy growth and development, evidence shows that unremitting psychosocial stress precipitates poor mental health and increases an individual's susceptibility to various morbidities (McEwen, 1998; Chrousos, 2009). Of note, recent evidence demonstrates a strong associative link between chronic stress and CVD (Rosengren *et al.*, 2004; O'Donnell *et al.*, 2016; Esler, 2017).

The most prevalent modern-day stressors are job strain, social isolation and personal or family burdens (Nyberg *et al.*, 2013; Non *et al.*, 2014; Wilson *et al.*, 2014). As these stressors are non-physical, our body's inherent stress response is largely rendered inappropriate. Despite the remarkable ability of animals to adapt and habituate to challenging stimuli, research indicates that perpetual psychosocial stressors chronically upregulate stress-response mechanisms. This leads to disrupted endocrine and autonomic function which in turn advances behavioral and cardiovascular derangements (Agorastos *et al.*, 2018).

Chronically stressed individuals typically present with elevated circulating levels of glucocorticoids and catecholamines. These effectors exert profound cardiovascular, metabolic and immunologic affects (Charmandari *et al.*, 2005). Given the proximity of these hormonal factors to the endothelium as well as the sensitivity of endothelial cells to hematologic and inflammatory alterations, it is not surprising that this single-cell layer has emerged as a particularly susceptible target of perturbed neuroendocrine function (Golbidi *et al.*, 2015). Furthermore, endothelial dysfunction, which is characterized by reduced NO levels, represents an important step in the pathogenesis of atherosclerosis amongst other vascular aberrations (Yao *et al.*, 2019).

The use of appropriate animal models is crucial for investigating linking mechanisms between chronic stress and vascular disease (Neumann *et al.*, 2011; Scharf and Schmidt, 2012). The Unpredictable Chronic Mild Stress (UCMS) model is regularly used to induce chronic stress and

depressive-like behaviors in rodents. The reproducible capacity of this model to elicit behavioral and neuroendocrine symptoms similar to those of depressed individuals is testament to its validity and reliability (Frisbee *et al.*, 2015; Antoniuk *et al.*, 2019).

Although the behavioural alterations induced by the UCMS model are well established, few studies have employed this model to interrogate the link between chronic stress and endothelial dysfunction. In light of this, we subjected male Wistar rats to 8 weeks of UCMS to test our hypothesis that non-habituating stressors would impair endothelial function as a result of a dysregulated HPA axis and SNS. We further hypothesize that attenuated endothelium-dependent dilation would be accompanied by increased oxidative stress.

1.1 Hypothesis, aims and objectives summary

We hypothesize that eight weeks of UCMS would lead to 1) dysregulated HPA axis and SNS, 2) impaired endothelial function and 3) increased oxidative stress.

Aims:

1. Identify perturbations in HPA axis and SNS activity.
2. Determine whether endothelium-dependent vascular reactivity is impaired.
3. Measure vascular oxidative stress levels.

Objectives:

1. Assess the plasma concentration of various stress hormones: ACTH, corticosterone, epinephrine.
2. Measure the endothelial-dependent response to vasoconstrictors and vasodilators.
3. Investigate the plasma concentration of ET-1, a systemic marker of endothelial damage.
4. Determine activity of oxidant enzymes SOD and NOX.

2. Methods and materials

2.1 UCMS protocol and experimental design

The Unpredictable Chronic Mild Stress (UCMS) model was established as a translationally-relevant protocol for inducing physiological, neurobiological and behavioural symptoms that are clinically associated with chronic stress and depression (Frisbee *et al.*, 2015; Antoniuk *et al.*, 2019). This section details our adaption of the UCMS model.

The aim of this study was to investigate the effects of chronic stress on the cardiovascular system, with emphasis placed on assessing endothelial integrity and functionality. Two distinct experimental runs of the UCMS protocol were conducted. The aim of the first experiment (joint research work completed by Lucien Sher and Lukas Olivier) was an attempt to establish the UCMS in our laboratory and successfully induce a phenotype of chronic stress in male Wistar rats. Following various oxidative stress tests and molecular analyses, we were unable to confirm the successful implementation of the model, as no significant differences were observed between groups (refer Supplementary Data). We subsequently adapted the protocol to ensure the successful establishment of the model with a second experimental run. Although both protocols were executed in the same animal laboratory, the duration of some stressors was increased and now conducted during the sleep phase (versus awake phase for first run). While the experimental procedure of each protocol is described in detail below, the results of the second experiment will be the focus of this study.

At the end of the second UCMS protocol, endothelial functional assessments were performed on harvested rat aortas and additional molecular analyses included: a) measuring the plasma concentration of various stress-linked hormones, b) assessing the degree of SOD and NOX activity and c) probing for markers of endothelial damage. Collectively we aimed to gain a better understanding of how the chronic upregulation of the HPA axis and the SNS may contribute to endothelial dysfunction within an *in vivo* setting. As chronic stress is an understudied risk factor

for CVD onset, the current investigation should provide foundations for future molecular mechanisms to be explored.

2.1.1 Animals and ethics statement

The animals in this study were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Academy of Science (National Institute of Health publication No. 85-23, revised 1996). This study was performed with the approval of the Animal Ethics Committee of Stellenbosch University, (Ethics #: ACU-2018-6311) (Appendix A).

2.2 Experimental procedures

First experimental run: For this experiment, 18 male Wister rats were obtained (~200-250 g) and randomly divided into two groups, namely: Control and Stress (n = 9 per group). All rats were housed one-per-cage and in the same room for the duration of the entire protocol. Both groups experienced a 12-hour day-night cycle and had *ad libitum* access to standard chow and water. The rats were afforded one week to acclimatize to the investigators and their surrounding environment. Following the acclimatization period, the UCMS protocol was initiated (Figure 4). The Control group was largely left alone and did not experience any stressors (as detailed for the UCMS protocol) for the duration of the study (eight weeks). By contrast, the Stress group was exposed to one stressor per day, lasting either four or eight hours (depending on the stressor), for six days per week. Such stressors were applied during the awake phase and were chosen in a random fashion to minimize habituation (Table 3). Rats were stressed during the awake phase to better simulate the human context. Body weight and food consumption were measured on the seventh day of each week (Figure 4).

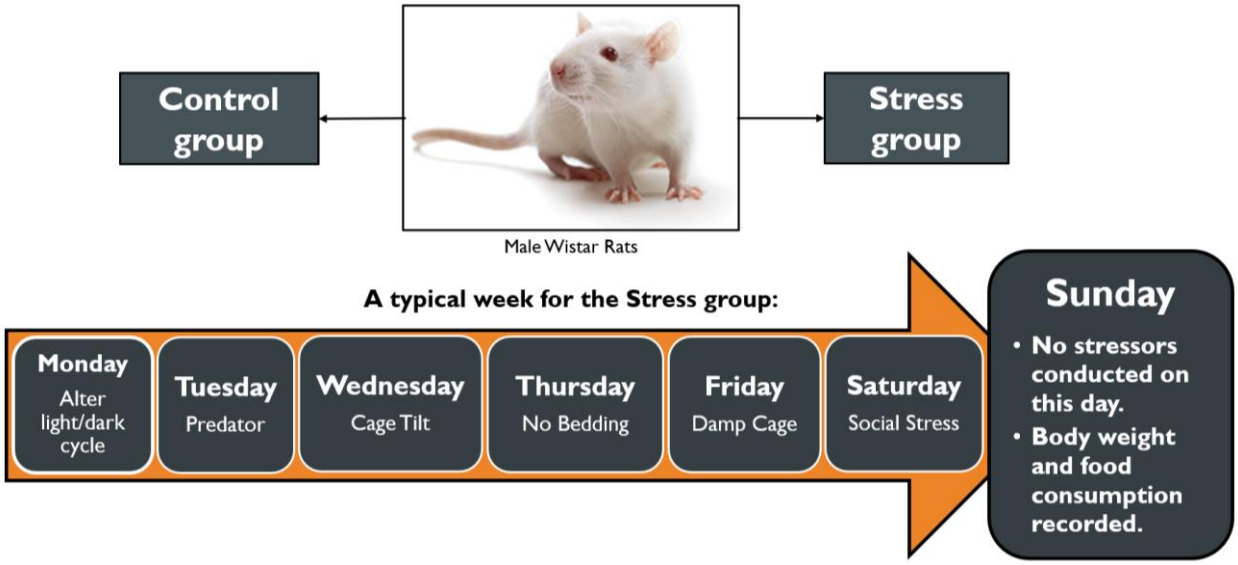

Figure 4: Schematic representation of the experimental procedure.

Second experimental run: For this experimental procedure, 32 male Wistar rats (~200-250 g) were randomly divided into three groups: Control (n = 12), Stress (n = 12) and Negative Control (n = 8). Unlike the first experimental run, the Control and Stress groups were now housed in separate rooms. Moreover, the Control group was housed two-per-cage and rats of the Stress group were housed individually. Control rats were housed in separate rooms to prevent the possibility of any communication between groups. Because rats are social animals, individual housing is a stressor in itself and for this reason the Control rats were now housed in pairs (Antoniuk *et al.*, 2019). Rats in the Negative Control group were housed four-per-cage in a third room. Following the acclimatization period, the Stress rat group was exposed to one random stressor per day, for a period of four to eight hours (depending on the stressor). Notably, such stressors were now applied during the sleep phase as indicated in the original UCMS protocol (Frisbee *et al.*, 2015). To increase stressor severity, the duration of the Damp Bedding and Cage Tilt stressors were extended to eight hours. For the same reason, cat litter was replaced with bobcat urine (The Pee Mart, Vassalboro ME) for the predator stressor (Table 3).

Table 3: Description of the UCMS stressors for each of the respective experimental procedures.

Stressor	Experimental Procedure 1 (Rats stressed during awake phase)	Experimental Procedure 2 (Rats stressed during sleep phase)
Damp Bedding	Approximately 500 mL of water was added to the rat's bedding in such a way to avoid its pooling within the cage (4 hours).	Stressor was not altered; however, duration increased to 8 hours.
No Bedding	The rat's bedding was removed from the cage for the duration of this stressor (4 hours).	Stressor was not altered.
Cage Tilt	This rat's cage was tilted at an angle of 45° and the bedding was removed for the entirety of this stressor (4 hours).	Stressor was not altered; however, duration increased to 8 hours.
Light/Dark	This stressor involves the disruption of the rat's light-dark cycle. During the awake phase, the lights were turned on and off, every 30 minutes, for a period of 8 hours.	Lights were turned on and off, every 30 minutes, for a period of 8 hours during the sleep phase.

Predator	The rats were exposed to cat litter that had been urinated on by a common house-cat (4 hours).	The rats were exposed to concentrated bobcat urine (liquid and granulated form) (4 hours).
Social Stress	All Stress rats had to spend the duration of this stressor in a neighboring rat's cage (4 hours).	Stressor not altered.

2.2.1 Weight and food consumption

The weight of the provided standard chow was determined before placement into cages. On the seventh day of each week, the remaining pellets were removed, weighed and the difference between the two pellet weights indicated the amount of food consumed per cage. The average weekly food consumption was then extrapolated from this data. This was carried out for the duration of the study.

2.2.2 Blood and tissue collection

Rats were anesthetized using 5% isoflourane gas (Piramal, Bethlehem PA) a week prior to the end of the second experimental run. Approximately 1.5 mL of blood was drawn from the right or left common carotid artery and immediately transferred into ethylenediaminetetraacetic acid (EDTA) vacutainers (BD, Franklin Lakes NJ). Subsequent to the rats being placed on their back, a hypodermic needle was inserted in the space between the sternum forelimb to pierce the carotid artery. Within 30 minutes of collection, blood was centrifuged at 1, 000 *x g* for 15 minutes at 4°C (Boeco M240, Hamburg, Germany). The supernatant was then transferred into fresh microfuge tubes and stored at -80°C for later analysis.

At the end of the second experimental run, rats were euthanized with an intraperitoneally administered overdose of sodium pentobarbital (Piramal, Bethlehem PA). Following excision of the heart, blood was collected in EDTA vacutainers (BD, Franklin Lakes NJ) and centrifuged at 1, 000 *x g* for 15 minutes at 4°C (Boeco M240, Hamburg, Germany). The supernatant was then transferred into fresh microfuge tubes and stored at -80°C for later analysis. Following blood collection, the aorta was immediately dissected and transferred to ice-cold Krebs-Henseleit (KH) buffer (NaCl 119 mM, $NaHCO_3$ 24.9 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $MgSO_47H_2O$ 0.59 mM, Na_2SO_4 0.59 mM, $CaCl_2H_2O$ 1.25 mM and glucose 10 mM). Together with plasma, the remaining organs were harvested and stored at -80°C for further molecular analyses.

2.3 Analyses

A variety of analyses were performed on aortic tissue and plasma to identify differences in HPA axis and SNS activity and perturbations in endothelial function. These included enzyme-linked immunosorbent assays (ELISAs), vascular function analyses and oxidative stress tests.

2.3.1 ELISAs

ELISAs were obtained (Elabscience Biotechnology Inc, Houston TX) for the determination of plasma ACTH (E-EL-R0048), corticosterone (E-EL-R0269), E (E-EL-0045) and ET-1 (E-EL-R1458) concentrations (Appendix B). Here, the ACTH and corticosterone assays were both performed on blood drawn from the carotid artery whereas the E and ET-1 ELISAs were conducted on blood drawn following excision of heart.

The standard working solution, biotinylated detection antibody and sample were incubated for 45 minutes at 37°C in a 96-well plate that had been pre-coated with either rat ACTH, corticosterone, E or ET-1 antibodies (Ab). During the reaction, the hormone to be measured in the sample, competes with a fixed number of hormone-specific sites on the biotinylated detection Ab. Excess unbound sample or standard was washed from the wells before horseradish peroxidase (HRP) was added and the plate incubated at 37°C (see Appendix B for incubation times). Substrate for

the HRP enzyme was then added before the reaction was terminated by the stop solution. The color change was measured spectrophotometrically at a wavelength of 450 nm (EZ Read 400 Microplate reader, Biochrom, Holliston MA). Hormone concentrations were then extrapolated from these data (Appendix B).

2.3.2 Vascular function analyses

All reagents were prepared each day according to standard protocols (Appendix C). Following euthanasia, harvested aortas were immediately arrested in ice-cold KH buffer (Appendix C). Excess connective and adipose tissue were then carefully removed from the exterior vessel wall before the aortic ring (3 mm) was mounted on the rig (Biopac Systems Inc., Goleta CA). The aortic ring was then lowered into KH buffer (NaCl: 119 mM, $NaHCO_3$: 24.9 mM, KCl: 4.7 mM, KH_2PO_4: 1.2 mM, $MgSO_47H_2O$: 0.59 mM, Na_2SO_4: 0.59 mM, $CaCl_2H_2O$: 1.25 mM and glucose: 10 mM), maintained at approximately 36.5-37°C and gassed with 95% O_2 and 5% CO_2.

Tension was gradually increased to from 0 to 1.5 g over a total period of 30 minutes, with buffer changes at 10 and 20 minutes respectively. Next, 2.5 µL of 1 mM phenylephrine (Phe) stock solution (Appendix C) was then added to 25 mL of KH buffer to induce aortic vasoconstriction. Once maximal contraction had been achieved, 25 µL of a 10 mM acetylcholine (ACh) stock solution (Stock A) was then administered to the water bath. Following maximal dilation, the aorta was washed 3 times with pre-warmed KH buffer and stabilized at 1.5 g for another 30 minutes, with washing steps at 10 and 20 minutes respectively. Cumulative concentrations of Phe followed by cumulative concentrations of ACh were then added (Mudau *et al.*, 2012) (Table 4).

Table 4: Cumulative doses of the respective drugs administered.

1. Cumulative doses of Phe	2. Cumulative doses of ACh
Add 2.5 µL of 1 mM stock solution.	Add 7.5 µL of a 1 mM solution (Stock C)

Add 5 µL of 1 mM stock solution.	Add 17.5 µL of Stock C.
Add 5 µL of 1 mM stock solution.	Add 42.5 µL of Stock C.
Add 7.5 µL of 1 mM stock solution.	Add 14.3 µL of a 100 µM solution (Stock B)
Add 5 µL of 1 mM stock solution.	Add 220 µL of Stock B.

The experiment was concluded once maximal vasodilation had been achieved. Results were analyzed using AcqKowledge Software (Biopac Systems Inc., Goleta CA) and the remaining aortic tissue was stored at -80°C for later molecular analysis.

2.3.3 Oxidative stress analyses

Pulverised aortic tissue was combined with 500 µL of phosphate-buffered saline (PBS) (pH 7.4) in 2 mL microfuge tubes (Appendix D). The tissue was further homogenized by adding 7 stainless steel beads (1.6 mm) (Next Advance, Troy NY) to the tubes and, using a Bullet Blender (Next Advance, Troy NY) (speed setting of 8) for 5x one-minute periods, with a minute interval in between. The homogenate was then centrifuged (Boeco M240, Hamburg, Germany) for 20 minutes at 4°C at 21, 950 *x* g. The supernatant was subsequently transferred into fresh microfuge tubes and stored at -80°C for later analyses.

2.3.3.1 SOD assay

To measure the concentration of SOD, 12 µL of sample was combined with 15 µL of 1,6-hexanediol and 53 µL of SOD phosphate buffer in a clear 96-well plate (Appendix D). Next, 170 µL of DETAPAC (Diethylenetriaminepentaacetic acid) was then added to initiate the reaction which was measured at a wavelength of 490 nm for five minutes (Multiskan Spectrum, Thermo Electron corporation, Waltham MA). Superoxide dismutase is known to inhibit the reaction between 1,6-hexanediol and DETAPAC and the absorbance is directly proportional to the level of

reaction inhibition. The concentration of SOD was then determined using the generated absorbance readings (Appendix D).

2.3.3.2 NADPH oxidase assay

The degree of NOX activity was determined using a modified assay (Abid *et al.*, 2007) (Appendix D). A luminometer was used to measure the degree of emitted light generated when NADPH oxidase complex donates electrons to lucigenin. An assay buffer containing 120 mM NACl, 250 mM HEPES (pH 7.4), 5.9 mM KCl, 11 mM glucose, 1.75 mM $CACl_2(2H_2O)$, 0.5 mM EDTA, 100 µM NADH and 5 µM of lucigenin was combined with 100 µL of sample in a 96-well plate. Light emission was then detected over a period of 25 minutes with a Glomax-96 luminometer (Promega, Madison WI). Readings were then expressed as enzyme units (U)/mL (Appendix D).

2.4 Statistical analysis

All statistical analyses were performed using Statistica 13.0 (StatSoft Inc., Dell Software, Tulsa OK) and done in conjunction with Prof. Martin Kidd at the Centre for Statistical Consultation at Stellenbosch University. Body weight, percentage growth and food consumption data were analyzed with a repeated-measures analysis of variance (ANOVA) test. All normally distributed data comparing Control vs. Stress was evaluated using an unpaired t-test. Non-parametric data was analyzed using a Mann-Whitney test. A two-way ANOVA with Bonferroni post-hoc analysis was used to evaluate all endothelial functional data. Linear regression analyses were also performed on data generated from endothelial function assessments. A P-value <0.05 was considered significant and any outliers were excluded by means of a Grubbs test. All data is presented using Graphpad Prism 5.01 (Graphpad Software Inc, San Diego, CA) as mean ± standard error of the mean (SEM).

3. Results

Part of this study was completed in conjunction with another MSc student in our department (Lukas Olivier). Consequently, body weight (Figures 5 and 6) and food consumption (Figure 7) data were jointly generated by both investigators. Following completion of the UCMS protocol, blood from the first 6 rats of each group was designated to Lukas Olivier while the blood from the remaining 6 rats from each group was given to Lucien Sher for later analysis. Here, both investigators independently investigated the plasma concentration of various important stress hormones, namely: ACTH and corticosterone. Data sets generated from the ELISAs were reported both individually (Figure 8-9A) and in collaboration (Figure 8-9B) in order to increase sample size (from n = 6 to n = 12) and provide a more holistic depiction of the outcomes of the stress protocol. All other experiments were solely conducted by Lucien Sher (Figure 10 – 17). Of note, the Negative Control data was not included in any of the figures as we found no significant differences when compared to Controls.

3.1 Body weight

The body weight of each rat was recorded on a weekly basis (Figure 5) and expressed as a percentage of the rat's baseline weight (Figure 6). Although rats from the Control and Stress groups were age-matched, the Control group weighed significantly more than the Stress group for the duration of the study – to be discussed later. Notably, the rate of weight gain for the Stress group was higher than the Control group throughout the experiment and differed by 23 ± 3% at the end of the eight-week period ($p<0.05$ and $p<0.0001$) (Figure 6).

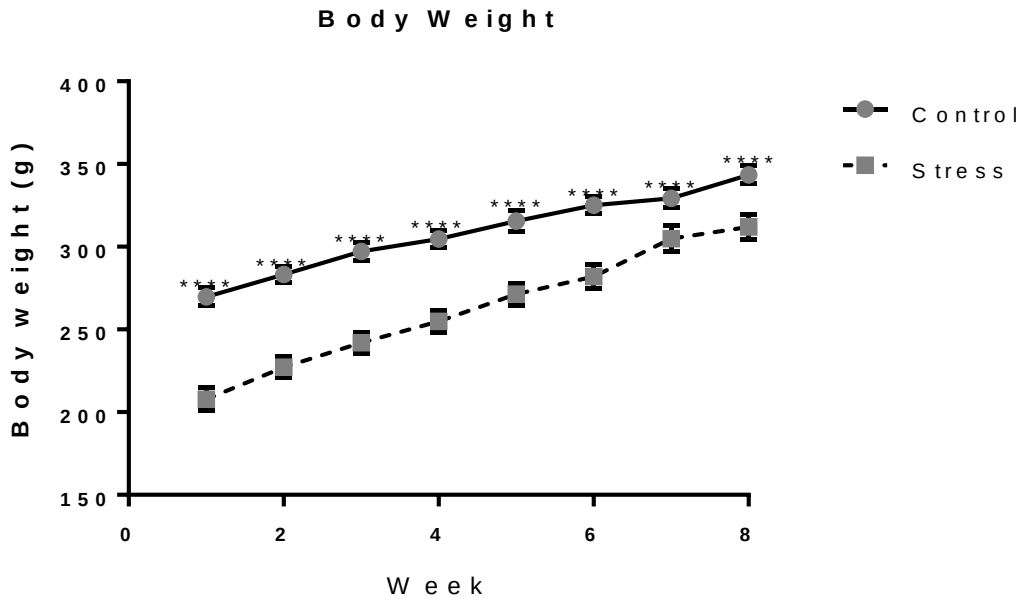

Figure 5: An assessment of body weights for Stress versus Control groups (data jointly generated in collaboration with Lukas Olivier). Data displayed as mean ± standard error of the mean (SEM); statistical analyses: repeated measures, two-way analysis of variance (ANOVA), Bonferroni post hoc; ****p<0.0001; n = 12.

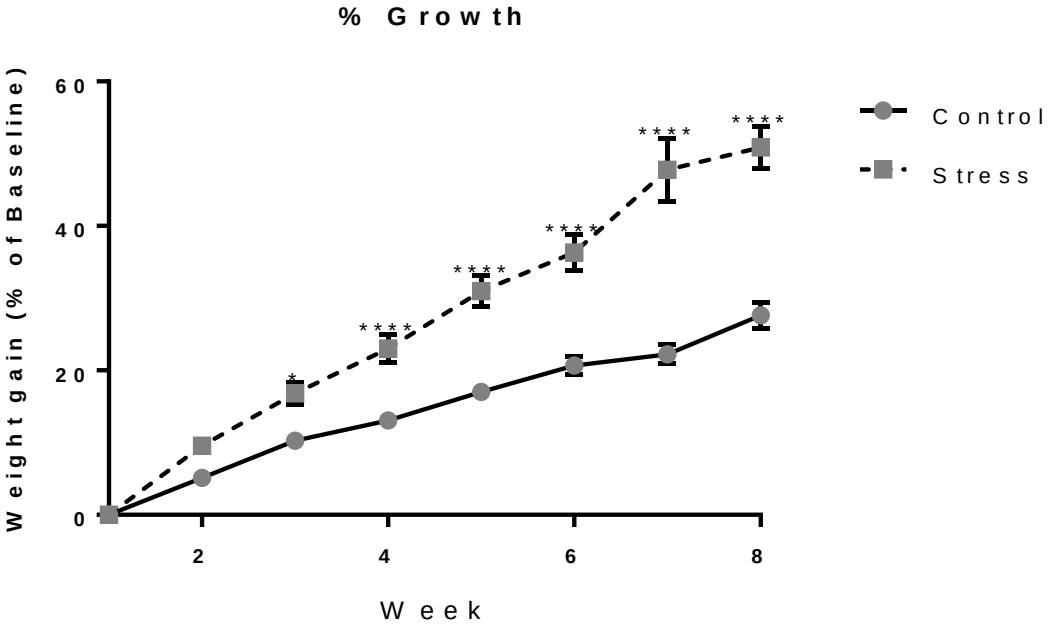

Figure 6: Percentage weight gain which was cumulative from the beginning of the study (data jointly generated in collaboration with Lukas Olivier). Data displayed as mean ± SEM; statistical analyses: repeated measures, two-way ANOVA, Bonferroni post hoc; *p<0.05, ****p<0.0001; n = 12.

3.2 Food consumption

At the end of each week the remaining pellets were weighed, and the amount of consumed standard chow was determined (Figure 7). The Control group ate significantly more than the Stress group at the end of the first week (p<0.05), while the Stress group consumed more food versus the Control group at weeks 5, 6 and 8 (p<0.05 and p<0.001) (Figure 7).

Figure 7: Food consumption for the Stress versus Control groups over the eight-week period (data jointly generated by Lucien Sher and Lukas Olivier). Data displayed as mean ± SEM; statistical analyses: repeated measures, two-way ANOVA, Bonferroni post hoc; *p<0.05, ***p<0.001; n = 12.

3.3 ELISAs

3.3.1 ACTH

Plasma ACTH levels were measured as marker of HPA axis activity and no differences were initially observed (Figure 8A). However, the combined data revealed attenuated ACTH levels in the Stress group when compared to the Control group (Figure 8B) (p<0.05).

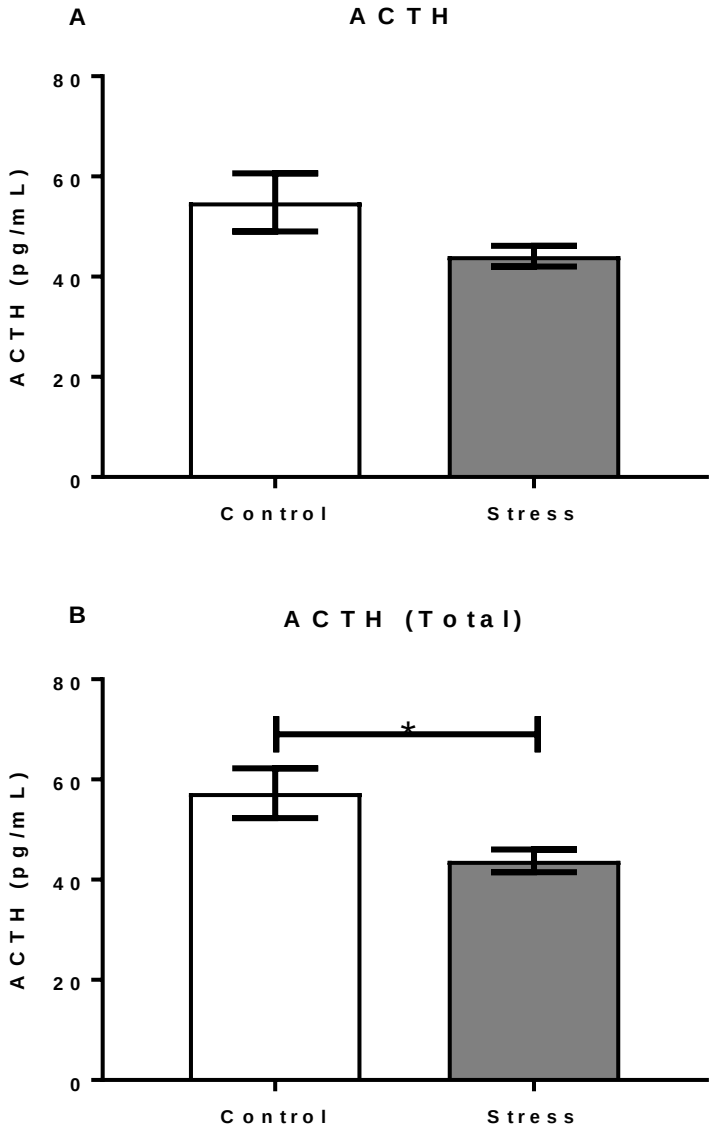

Figure 8: Plasma ACTH levels in response to stress. A - Data displayed as mean ± SEM; statistical analyses: unpaired t-test; Control and Stress: n = 6 (data generated by Lucien Sher). B – Data displayed as mean ± SEM; statistical analyses: unpaired t-test; *p<0.05; n = 12 (combined data: Lucien Sher and Lukas Olivier).

3.3.2 Corticosterone

Plasma corticosterone levels were also determined as it is one of the primary stress hormones and a reliable marker of HPA axis activity. No significant differences were initially observed (Figure 9A and 9B). To gain greater insights into these data, we also plotted the individual data points (refer black circles for Figure 9B). Interestingly, it seems that there are two distinct patterns, i.e. hyper- and hypo-responsive subgroups in response to the chronic stress protocol (Figure 9B). Although this phenomenon was maintained for the combined data (Figure 9B), no subgroups were identified for the other measurements (Figure 8 – 17).

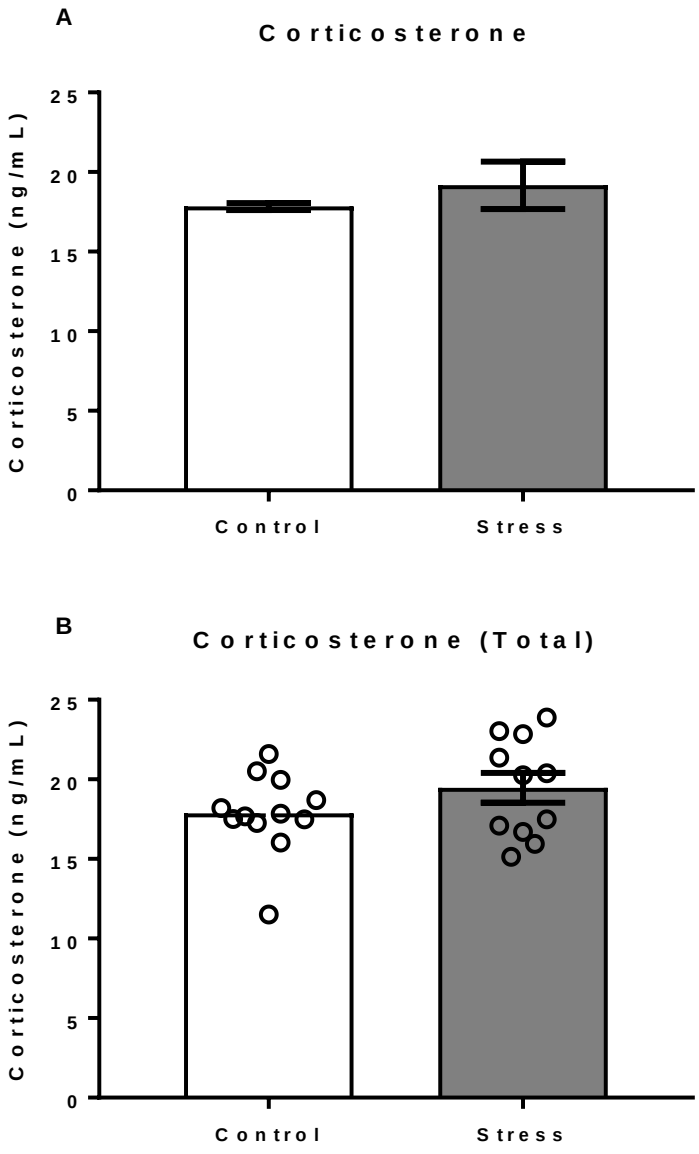

Figure 9: Plasma corticosterone concentrations in response to chronic stress. A **–** Data displayed as mean ± SEM; statistical analyses: unpaired t-test; n = 6 (data generated by Lucien Sher). B **–** Data displayed as mean ± SEM; statistical analyses: unpaired t-test. Control: n = 12 and Stress: n = 11 (combined data: Lucien Sher and Lukas Olivier).

3.3.3 Epinephrine

Plasma E concentrations were measured to gauge sympathomedullary activity. No significant differences in E levels were observed between groups (Figure 10).

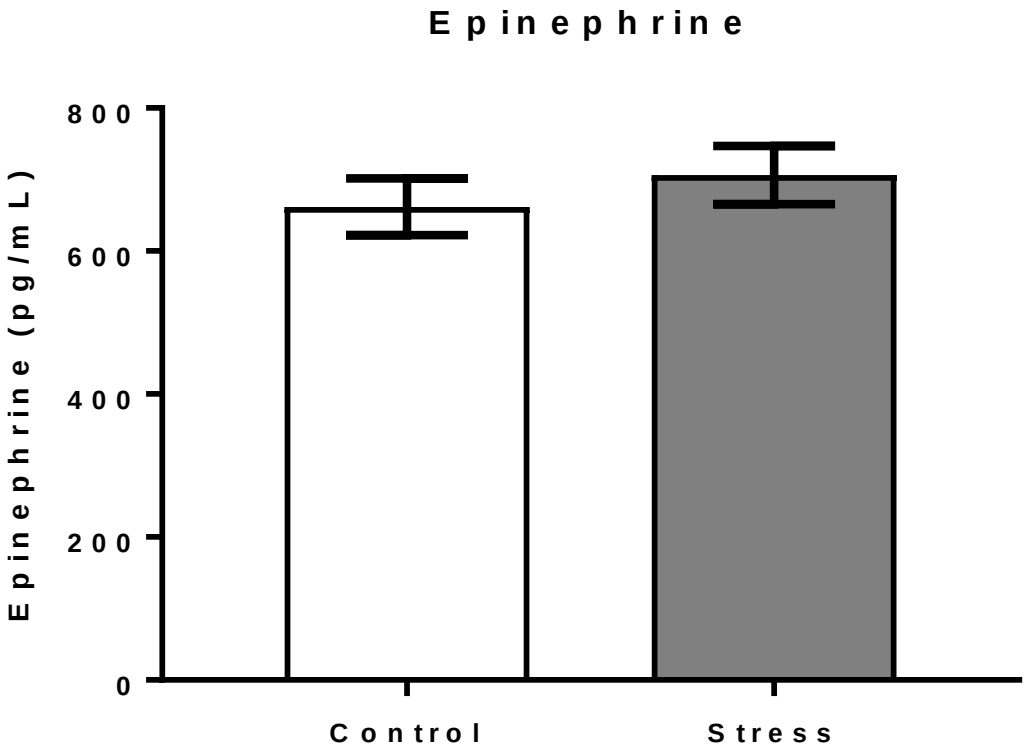

Figure 10: Plasma E concentrations in response to chronic stress. Data displayed as mean ± SEM; statistical analyses: unpaired t-test; n = 12 (data generated by Lucien Sher).

3.3.4 ET-1

ET-1 is released from the endothelium in response to endothelial damage or dysfunction (Fox *et al.*, 2018). No significant differences in plasma ET-1 expression were noted (Figure 11).

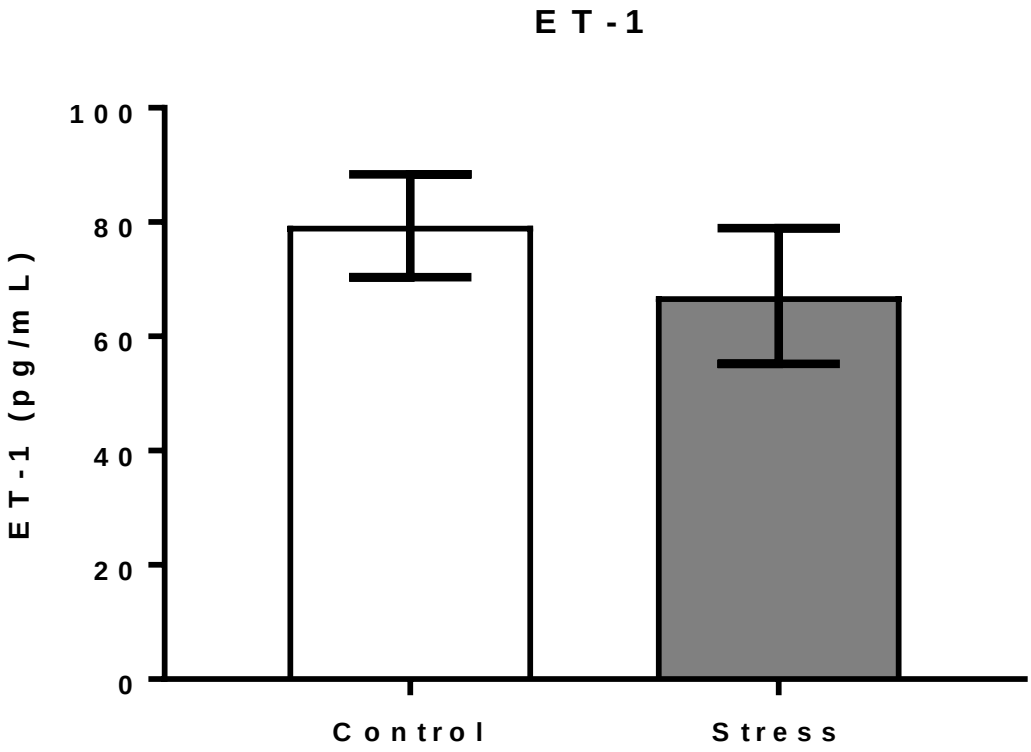

Figure 11: Plasma ET-1 levels in response to chronic stress. Data displayed as mean ± SEM; statistical analyses: unpaired t-test; n = 12 (data generated by Lucien Sher).

3.4 Endothelial function

Endothelial function was assessed by testing the contractile and dilatory ability of harvested aortic rings following exposure to Phe and ACh, respectively (Figure 12-16). Although no differences in the contractive ability of the aortic rings were noted (Figure 12 and 14), our results show that the dilatory ability of the Stress aortas was impaired when compared to Controls ($p<0.05$ and $p<0.005$) (Figure 13 and 15). Moreover, to achieve 50% relaxation, the Stress group required

significantly more ACh than the Control ($p<0.05$) (Figure 15). Overall, aortic rings from the Control group relaxed more than the Stress group following administration of the last dose of ACh ($p<0.05$) (Figure 16B).

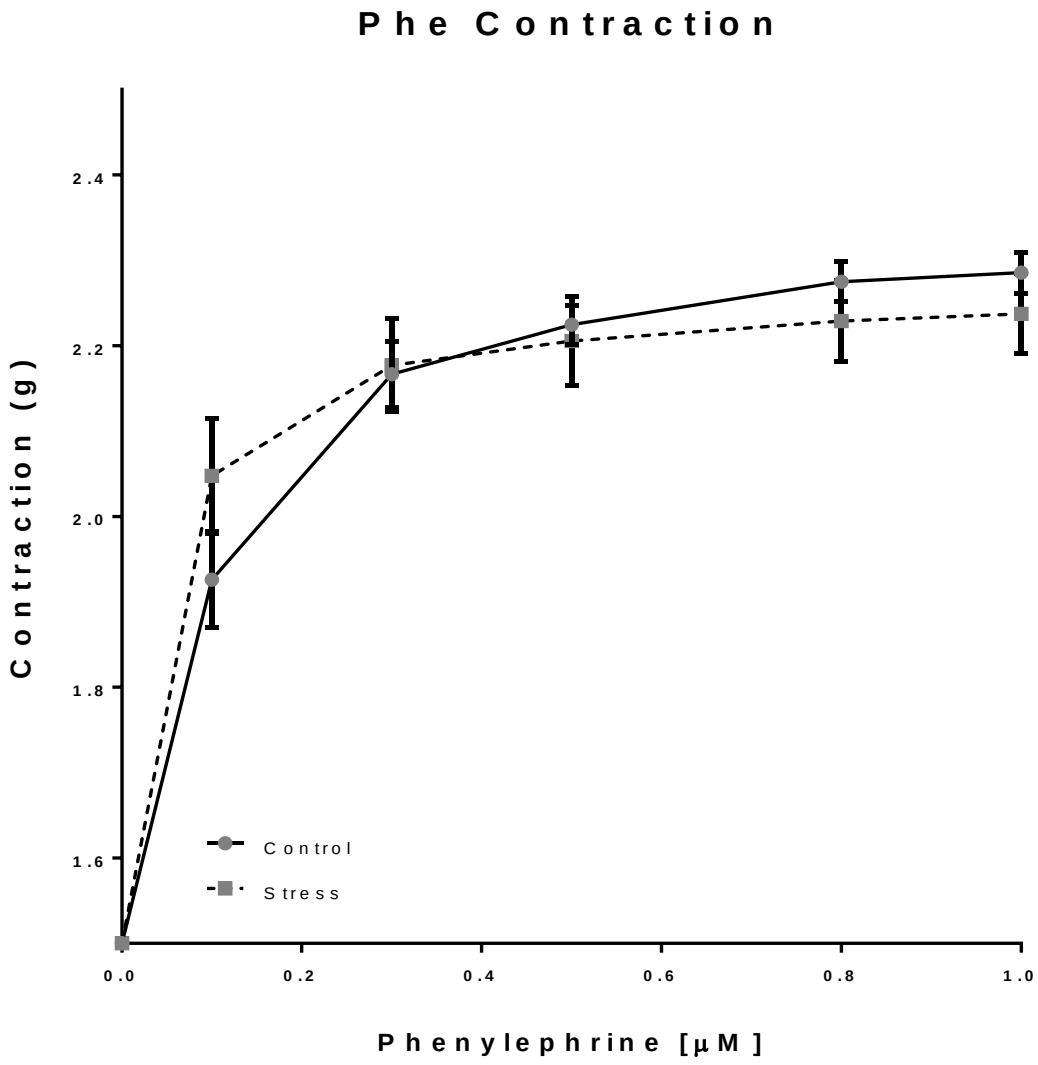

Figure 12: Harvested aortas exposed to cumulative doses of Phe to stimulate vasoconstriction. Data displayed as mean ± SEM; statistical analyses: repeated measures 2-way ANOVA, Bonferroni post hoc. Control: n = 7 and Stress: n = 11 (data generated by Lucien Sher).

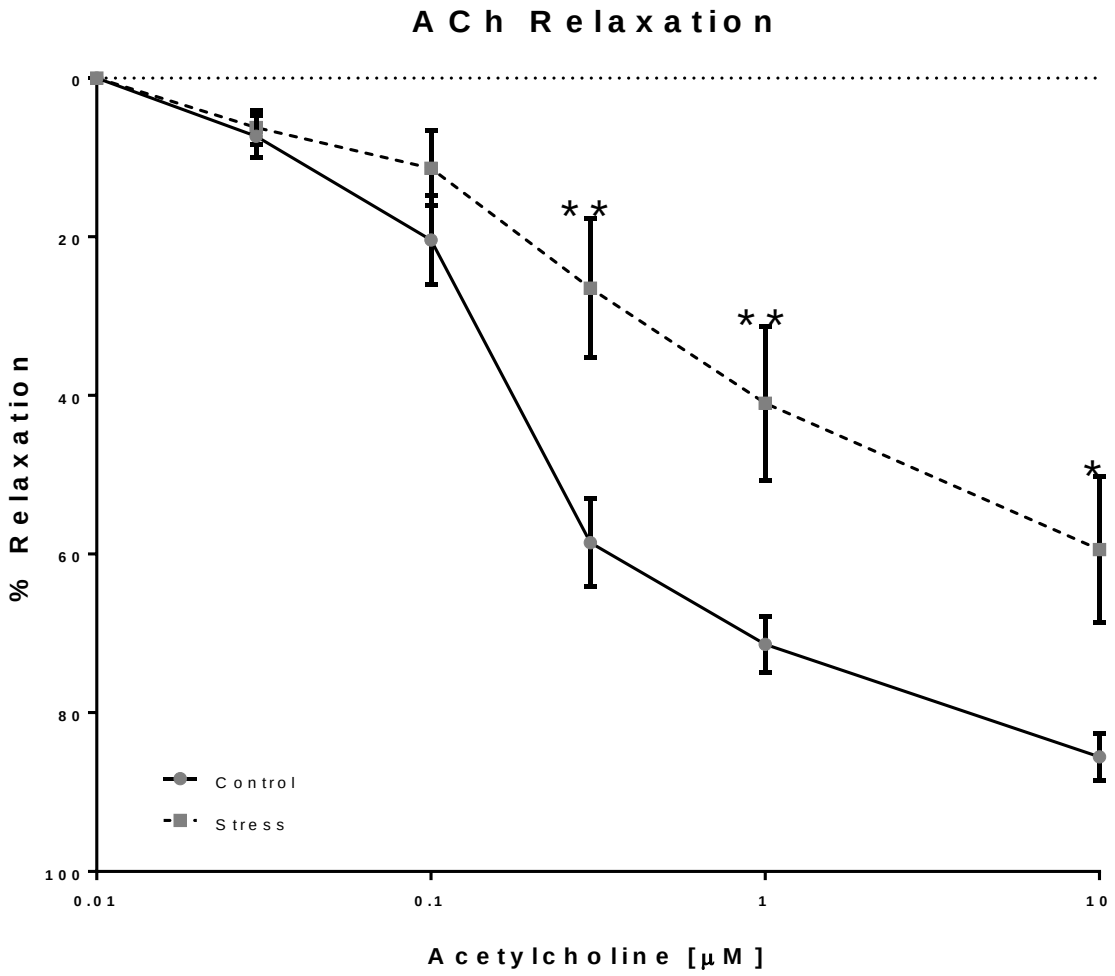

Figure 13: Administration of varying ACh concentrations to induce vasodilation of aortic rings. Data displayed as mean ± SEM; statistical analyses: repeated measures 2-way ANOVA, Bonferroni post hoc; *p<0.05, **p<0.005. Control: n = 7 and Stress: n = 11 (data generated by Lucien Sher).

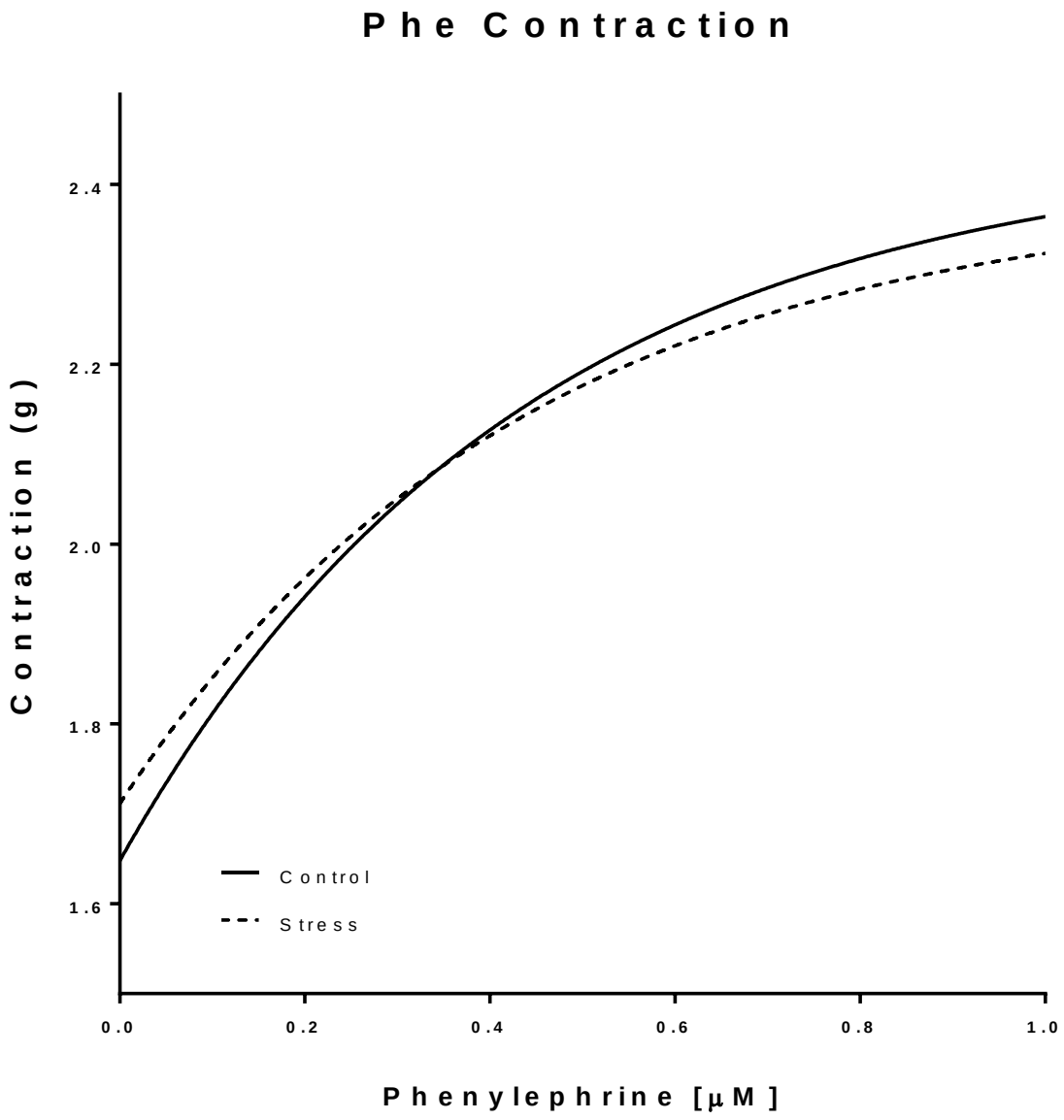

Figure 14: Nonlinear regression of Phe-induced vasoconstriction. Data displayed as mean ± SEM; statistical analyses: repeated measures 2-way ANOVA, Bonferroni post hoc; Control: n = 7 and Stress: n = 11 (data generated by Lucien Sher).

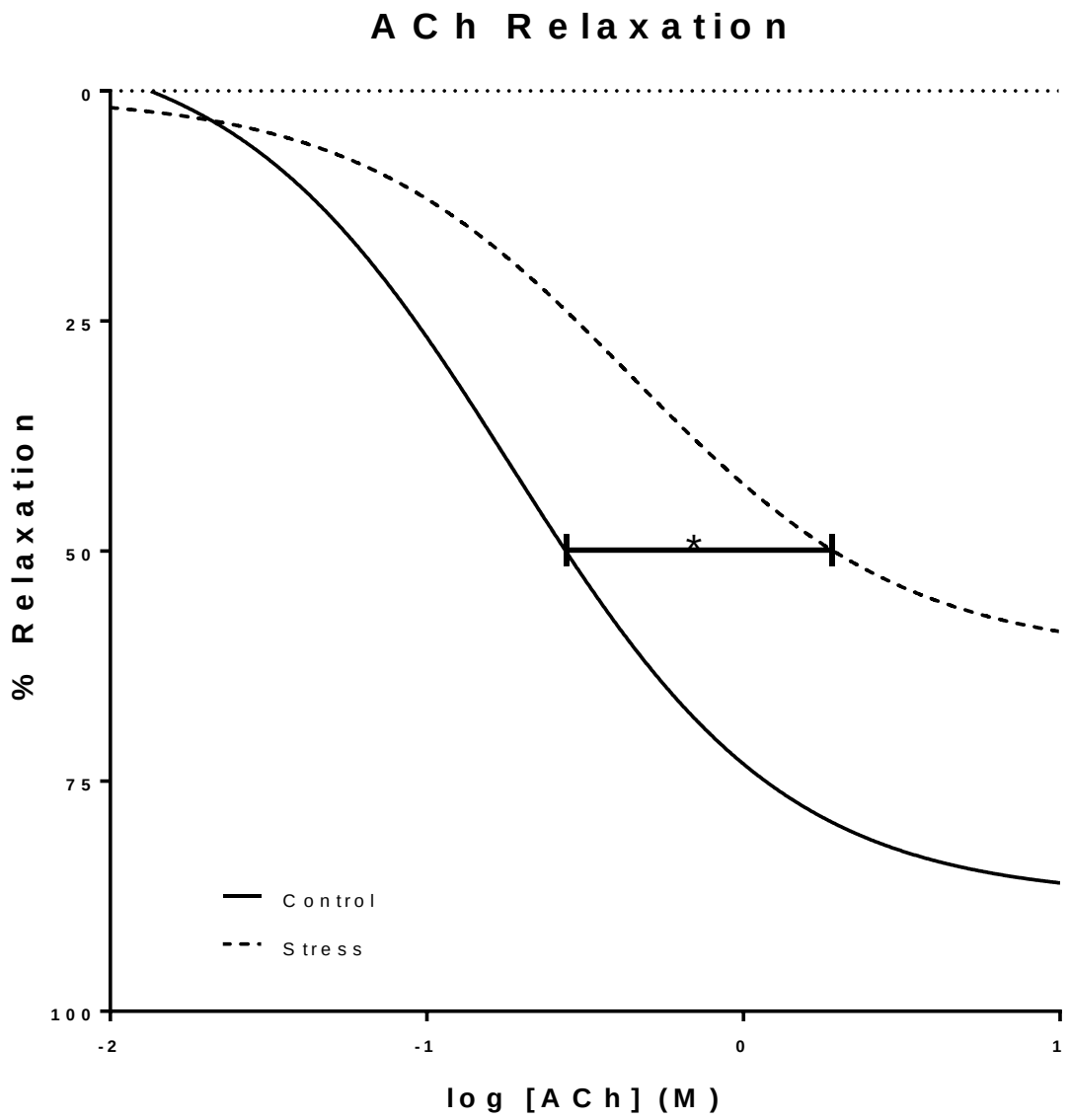

Figure 15: Nonlinear regression of ACh-induced vasodilation. Data displayed as mean ± SEM; statistical analyses: repeated measures 2-way ANOVA, Bonferroni post hoc; *p<0.05; Control: n = 7 and Stress: n = 11 (data generated by Lucien Sher).

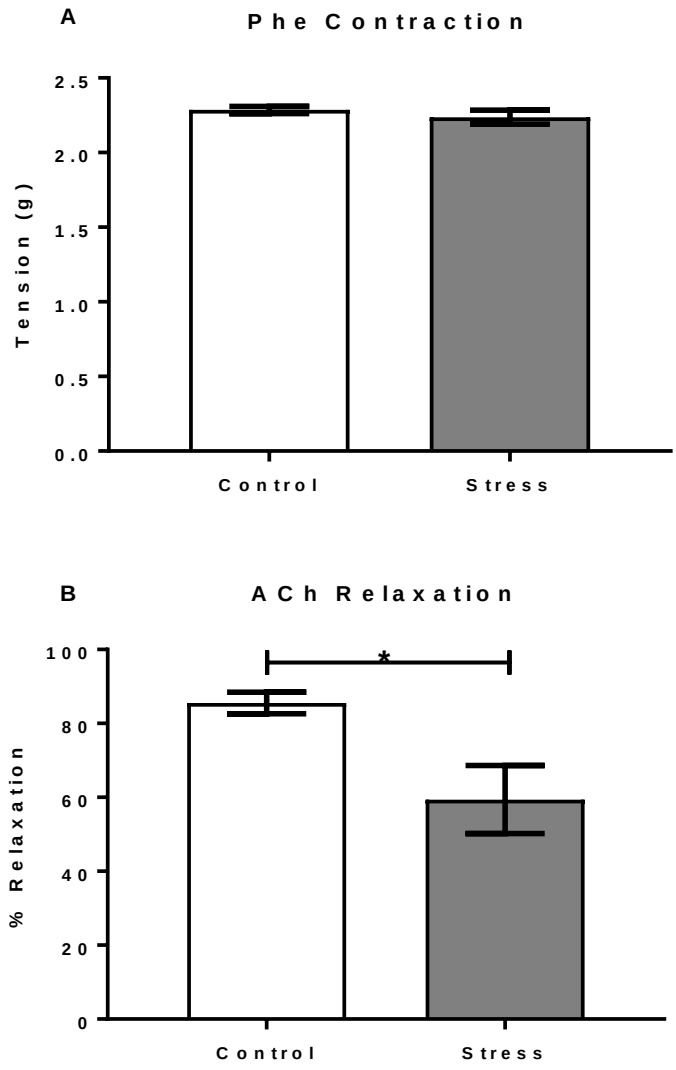

Figure 16: The degree of vasoconstriction (A) and vasodilation (B) after the final dose of the respective drugs were administered. All data displayed as mean ± SEM; statistical analyses: unpaired t-test; *p<0.05; Control: n = 7 and Stress: n = 11 (data generated by Lucien Sher).

3.5 Oxidative stress

Oxidative stress is one of the key mechanisms by which endothelial damage and dysfunction may arise following exposure to chronic stress. Superoxide dismutase catalyzes the formation of

oxygen and hydrogen peroxide from the superoxide molecule. Our data revealed that SOD activity was lower in the Stress group versus Controls ($p<0.05$) (Figure 17A). The activity of NOX was assessed as this enzyme is a key producer of ROS however, no differences in NOX activity were observed ($p = 0.13$) (Figure 17B).

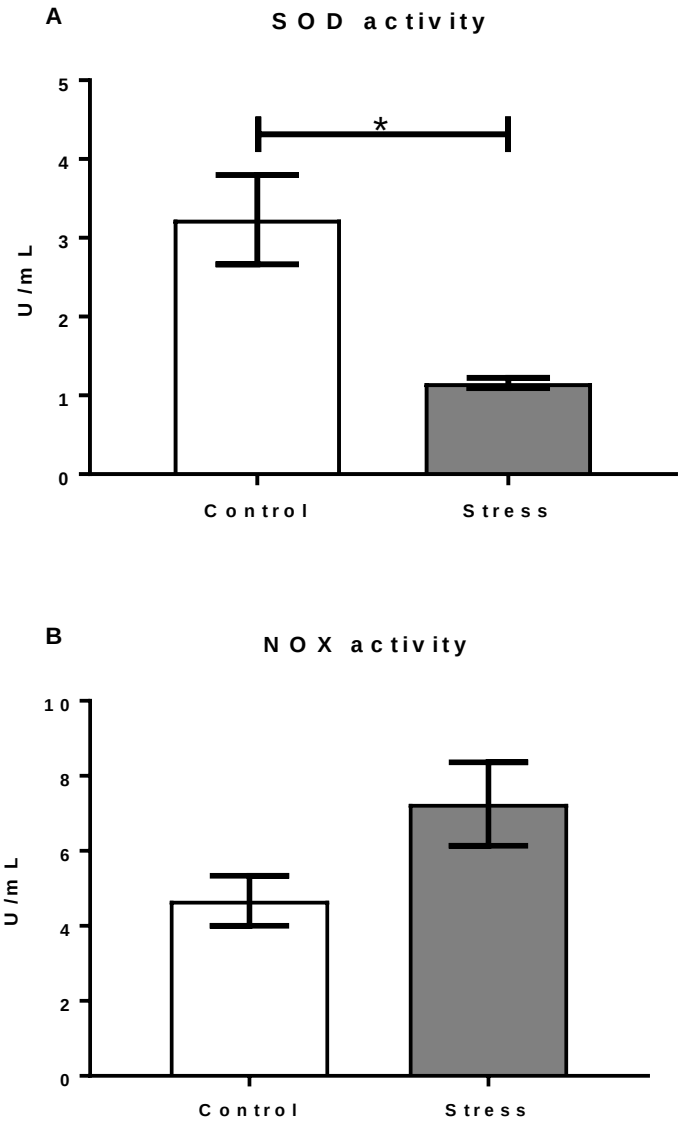

Figure 17: The activity of (A) SOD and (B) NOX in the thoracic aorta following chronic stress. All data displayed as mean ± SEM; statistical analyses: unpaired t-test; *$p<0.05$; $n = 4$.

4. Discussion

A large body of epidemiological and experimental evidence points towards a causative link between chronic psychosocial stress and the onset of CVD. However, limited knowledge exists regarding the molecular derangements that underpin the development of such cardiovascular pathologies. In light of this, we employed the UCMS model to further elucidate the underlying mechanisms whereby the sustained upregulation of stress-response systems may lead to compromised vascular function. In this study, two-months of UCMS resulted in 1) decreased plasma ACTH concentrations, 2) impaired endothelium-dependent dilation and 3) attenuated vascular SOD activity.

4.1 Efficacy of the UCMS model

The use of appropriate animal models is crucial for elucidating the negative effects of chronic stress on the cardiovascular system (Neumann *et al.*, 2011). Ideally these models should mimic the human context as accurately as possible, highlight pathological mechanistic contributors, elicit reproducible behavioural and physiological outcomes as well as provide opportunity for therapeutic intervention (Willner, 1997; Farooq *et al.*, 2012; Frisbee *et al.*, 2015). In light of these criteria, the efficacy of the UCMS model was validated by a number of studies (Table 5: studies 1-20). This model recruits appropriate neuroendocrine systems to induce behavioural and physiological adaptations analogous to those of patients suffering from poor mental health (Antoniuk *et al.*, 2019). Moreover, these symptoms are blunted following the administration of antidepressants, adrenergic receptor-blockers or natural remedies (Table 5: studies 1-2; 13-14; 16-19).

Behavioural adaptations and alterations in plasma or serological concentrations of stress hormones (corticosterone, ACTH, E) serve as the basis for confirming the successful implementation of the UCMS protocol (Frisbee *et al.*, 2015). Tests used to describe and measure depressive-like behaviours in rodents include the reward test, the splash test and the novelty

suppression test (Isingrini *et al.*, 2011). The reward test functions as a measure of anhedonia by demonstrating that repeated exposure to unpredictable stressors suppresses the acquisition of an appetite behaviour that is induced and maintained by a highly palatable food (Gambarana *et al.*, 2001). The splash test assesses standards of self-care and motivational behaviour by measuring the frequency and latency to initiate grooming after being sprayed by a 10% sucrose solution. Lastly, the novelty suppression test promotes a motivational conflict between eating behaviour and the fear of novelty. This is measured by the latency to initiate sniffing and eating of a food pellet (Ibarguen-Vargas *et al.*, 2008; Isingrini *et al.*, 2011).

Although the UCMS model represents a reliable tool for studying stress-mediated pathology, a high degree of variability exists across experimental studies (Table 5). This presents in the form of stressor regimen, method of euthanasia as well as animal species, strain and housing. Despite studies exposing animals to mild stressors in a randomized fashion, the number, character, severity and duration of UCMS stressors often differ. The length of the UCMS protocol may also differ with studies employing a varied experimental duration of 2-8 weeks (Table 5). For example, some even exposed mice to two separate stints of UCMS with a 6-week break in-between (Isingrini *et al.*, 2012). Animal housing is another important variable as rodents are social by nature. This means that individual housing is a stressor on its own. Contrastingly, housing two-or-more rats per cage provides camaraderie which negates the pathological effects of UCMS (Frisbee *et al.*, 2015).

Although a variety of different animals have been subjected to the UCMS protocol, the most common species employed are mice and rats. Rodent strains of note include Balb/c mice as well as Wistar-Kyoto and Sprague-Dawley rats (Table 5). A number of studies also employed apolipoprotein E-deficient ($ApoE^{-/-}$) mice to study the confounding effects of chronic stress on atherosclerotic plaque development (Table 5: studies 4-6). Species, strain and gender are important factors to consider as they can heavily influence the efficacy of the UCMS protocol (Wu

and Wang, 2010). For example, Balb/c mice are largely susceptible to the broad effects of chronic stress (Mineur *et al.*, 2006; D'Audiffret *et al.*, 2010), however they have intriguingly demonstrated particular resilience to developing vascular dysfunction (Friedman *et al.*, 2000; Miyoshi *et al.*, 2007). In line with this, d'Audiffret and colleagues noted variability in the pathological outcomes of vascular dysfunction in genetically identical mice subjects (D'Audiffret *et al.*, 2010). This suggests that individual responses to chronic UCMS may be heterogenous, even within a genetically identical strain. Furthermore, variable adaptions to challenging stimuli may lead to diverse avenues through which pathophysiological outcomes can precipitate.

Gender plays an equally important role, especially with regards to behavioural testing. For example, basal and stress-induced changes in sucrose consumption are differently expressed between male and female rats (Dalla *et al.*, 2005, 2008; Pitychoutis *et al.*, 2009). This may be a consequence of gender-orientated differences in taste (Clarke and Ossenkopp, 1998; Curtis *et al.*, 2004) or in response to a reward (Michaels and Holtzman, 2007). Overall, males present with decreased preference to sucrose following UCMS however, females seem to be more susceptible to UCMS in the context of other behavioural and biochemical tests (Dalla *et al.*, 2005, 2008).

Thus the ability to accurately study the associated-negative effects of chronic stress on the body, is heavily dependent on the adequacy of the model used to induce such stress. Although there are many different experimental protocols currently employed, the UCMS has emerged as a relevant and reliable tool for reproducing behavioural and physiological symptoms of poor mental health. Various observational (i.e. the reward and splash test) and molecular tests (i.e. plasma corticosterone levels) exist to confirm the successful implementation of chronic stress. Additionally, a large degree of variability exists amongst recent studies employing non-habituating stress protocols. This is important to acknowledge as it dilutes the applicability of findings and further complicates the contextualization of new observations.

With regards to our experiments, we initially stressed the rats during their awake phase to increase applicability to the human setting (Supplementary data, Figure 18-22). However, with the exception of attenuated antioxidant capacity (Figure 22), we observed no changes in our other biochemical measures. Although our second experimental run was largely similar to previous UCMS studies (Table 5), some differences in our protocol persisted. For example, we only stressed our rats once per day and employed a total of six stressors. Other UCMS protocols exposed their rodents to two stressors per day and utilized a minimum of eight stressors or more (Table 5: studies 1-4; 10; 13; 17-19). We did not perform behavioral assessments due to the extensive validation of the protocol however, some of our findings were unexpected (ACTH and corticosterone) and this emphasizes the importance of these tests. In light of this, we propose that it may be useful to do a comprehensive analysis of all studies completed thus far with the aim to develop a specific set of guidelines/standards for the UCMS model that in turn should enhance the successful translation of findings.

Table 5: Summary of findings from studies that employed the UCMS model. Unless otherwise stated, animals were stressed during their sleep phase (lights on). Stress groups were housed individually and animals had *ad libitum* access to standard chow and water.

Study #	Stressor regimen	Animals used	Investigated site	Observations	Reference
1	Mice were exposed to 11 different stressors (multiple stressors/day) for 7 weeks. Euthanasia: - Intervention: Agomelatine (10 mg/kg), melatonin (10 mg/kg) and fluoxetine (15 mg/kg) for 5 weeks.	Male inbred Balb/c ByJ mice (9-10 weeks old).	Plasma.	↑ ACTH; ↑ corticosterone, ↑ IL-6; ↑ TNF-α. Pharmaceutical interventions prevented behavioural deficits in grooming, attack frequency, and immobility time as well as increases in biochemical markers.	(Mutlu *et al.*, 2013)
2	Mice were exposed to 11 different stressors (multiple stressors/day) for 7 weeks. Euthanasia: -	Male inbred Balb/c ByJ mice (9-10 weeks old).	Plasma.	↑ ACTH; ↑ IL-6; ↑ TNF-α. Tianeptine, olanzapine and fluoxetine abolished stress-induced reductions in coat state, grooming and attack frequency. These drugs	(Mutlu *et al.*, 2012)

	Intervention: Tianeptine (5 mg/kg), olanzapine (2.5 mg/kg) and fluoxetine (15 mg/kg) for 5 weeks.			further prevented enhanced plasma ACTH, IL-6 levels and TNF-α levels.	
3	Rats were exposed to 7 different stressors (three stressors/day) times per day, for 21 days. Euthanasia: - Intervention: Electroacupuncture.	Male Wistar rats (150-170 g).	Plasma.	↑ ACTH; ↑ corticosterone. Intervention reduced behavioural and physiological markers of stress to normal.	(Liu *et al.*, 2013)
4	Mice subjected to unpredictable chronic stress regime (10 different stressors) for 14 days with one stressor/day. Euthanasia: -	Male ApoE$^{-/-}$ mice (20 weeks old) on Balb/c background.	Plasma, serum and brachiocephalic artery.	↓ Body weight; ↑ intimal VCAM-1 and ICAM-1 expression; ↑ C-reactive protein and IL-6; ↑ plaque size.	(Zhang *et al.*, 2010)
5	Mice exposed to five different stressors for 12 weeks (2 hours/day for 5 days/week).	Female ApoE$^{-/-}$ mice (C57BL/6 background).	Plasma, thoracic aorta and urine.	↓ Body weight; ↓ urinary corticosterone; no difference in plasma corticosterone	(Bernberg *et al.*, 2009)

	Housing: Individually or in groups of four. Euthanasia: Sodium pentobarbital.	Diet: standard diet or high salt diet.		levels; ↑ heart rate and blood pressure.	
6	Mice subjected to six different stressors for 1, 3 or 6 weeks (depending on strain). Housing: Multiple animals per cage (number unknown). Euthanasia: -	Female C57BL/6 and ApoE$^{-/-}$ mice (10-12 weeks old).	Plasma, aortic root and bone marrow.	No differences in body weight; ↑ leukocytes, monocytes and neutrophils in blood and in intima; ↑ NE.	(Heidt *et al.*, 2014)
7	Mice experienced 2-4 socio-environmental stressors/day for eight weeks. Euthanasia (ELISA): CO_2 asphyxiation and decapitation. Euthanasia (Vascular function): Sodium pentobarbital.	Seven-week-old male BALB/cJ mice.	Serum and thoracic aorta.	No differences in body weight; ↑ in total corticosterone levels; no differences in Phe-induced vasoconstriction; ↓ ACh-induced vasodilation and ↓ maximal response to ACh.	(Isingrini *et al.*, 2011)

8	Rats subjected to two weeks of randomized social stressors. Housing: Five rats per cage. Euthanasia: CO_2 anesthesia before decapitation.	Male borderline hypertensive rats and normotensive Wistar-Kyoto rats (5-weeks-old).	Plasma, brain and thoracic aorta.	↑ Plasma corticosterone; ↓ brain and cardiac NO synthesis; initially no differences in aortic NO ACh-dependent vasodilation however, two-weeks post stress: ↓ aortic NO production and ACh-dependent dilation.	(Bernatova *et al.*, 2018)
9	Exposure to seven different stressors for eight weeks. Euthanasia: Sodium pentobarbital.	9-week-old male BALB/cJ mice.	Plasma and thoracic aorta.	No differences in Phe-induced vasoconstriction; ↓ methacholine-induced vasodilation; no differences in arterial expression of eNOS; ↓ NO levels, ↑ H_2O_2 levels; ↑ insulin.	(D'Audiffret *et al.*, 2010)

10	Mice exposed to 4 weeks of UCMS protocol (8 stressors). Euthanasia: Sodium pentobarbital. Intervention: iNOS inhibitor.	9-week-old male BALB, cJ mice (20-25 g).	Plasma and brain.	↑ Levels of iNOS mRNA in cortex, ↑ nitrite in plasma, ↓ Nissl bodies; ↑ neuronal cell damage. Effects ameliorated with iNOS inhibitor.	(Peng *et al.*, 2012)
11	Rats experienced 6 different stressors for 3 weeks of an 8-week period. Rats euthanized 15 days after the final stressor. Euthanasia: Decapitation.	Male Sprague-Dawley rats (60 days old; weighing 300-350 g).	Plasma and thoracic aorta.	↑ Plasma corticosterone, ↑ sensitivity to Phe-induced vasoconstriction.	(Neves *et al.*, 2009)
12	Rats subjected to 8 weeks of UCMS (7 different stressors). Euthanasia: Sodium pentobarbital.	8-week-old male and female Zucker rats.	Plasma and thoracic aorta.	Both males and females demonstrate ↓ vasodilation following methacholine, ↓ NO levels and ↑ H_2O_2 in vasculature; ↑ corticosterone	(Brooks *et al.*, 2018)

				levels in both males and females.	
13	Rats exposed to 8 weeks of UCMS (9 different stressors). Euthanasia: - Intervention: Etanercept.	Adult male Wistar rats (250-300 g).	Thoracic aorta.	↓ Body weight; no differences in blood pressure; ↓ eNOS; ↓ endothelium-dependent dilation. Effects abolished by etanercept.	(Bayramgurler *et al.*, 2013)
14	Mice subjected to two, 2-week UCMS protocols (5 different stressors), separated by an interval of 6 weeks. Euthanasia: Sodium pentobarbital. Intervention: Fluoxetine.	Male BALB/c mice (7-9 weeks old).	Thoracic aorta.	No differences in body weight; ↑ depressive behaviour; ↓ NO-dependent relaxation; ↑ atheroma. Effects abolished by fluoxetine treatment.	(Isingrini *et al.*, 2012)
15	Rats exposed to 6 different stressors, twice a day, for 6 weeks. Housing: -	Male and female Wistar rats.	Plasma and serum.	↑ NO levels in females compared to males; females had ↓ ICAM-1 levels, ↑ GPx activity and ↓ SOD activity	(Kamper *et al.*, 2009)

	Euthanasia: Decapitation			compared to males; ↑ malondialdehyde.	
16	Mice were subjected to variable stressors (one stressor/day) for 21 days. Euthanasia: Decapitation. Intervention: Bilberry extract, fluoxetine (10 mg/kg), L-arginine (125 mg/kg) and L-NAME (10 mg/kg) were administered daily for 21 days.	Male Laca mice (20–30 g).	Brain.	↑ Lipid peroxidation; ↑ nitrite; ↓ glutathione; ↓ SOD; ↓ catalase. Bilberry extract reversed expression of these parameters to baseline.	(Kumar *et al.*, 2012)
17	Mice were exposed to 12 different stressors for 6 weeks. Euthanasia: - Intervention: Liquiritigenin (7.5 and 15 mg/kg) and fluoxetine (20 mg/kg).	Male ICR mice (20–22 g).	Serum and hippocampus.	↑ Corticosterone; ↓ glutathione; ↓ SOD. These parameters returned to baseline following administration of liquiritigenin and fluoxetine.	(Tao *et al.*, 2016)

18	Mice experienced 9 different stressors (one stressor/day) for 2 weeks. Euthanasia: Decapitation under anesthesia. Intervention: Methyl jasmonate (5, 10 and 20 mg/kg).	Male Swiss mice (23.5 ± 1.5 g).	Serum and brain.	↑ Corticosterone; ↓ glutathione; ↓ SOD; ↑ malondialdehyde (MDA); ↑ TNF-α. Intervention improved biochemical measures.	(Adebesin *et al.*, 2017)
19	Rats were subjected to 10 different UCMS stressors (1-3 stressors/day), for 6 weeks. Euthanasia: Anesthetized and transcardially perfused. Intervention: Argan oil.	Male and female Wistar rats (34 ± 2 g; 21 days old).	Plasma, amygdala, hippocampus and frontal cortex.	↑ Corticosterone; ↑ NO; ↑ MDA; ↓ SOD; ↓ catalase; ↑ lipid peroxidation Argan oil returned all behavioural and physiological measurements to normal.	(Hicham *et al.*, 2018)
20	Rats subjected to 10 days of UCMS (6 different stressors). Euthanasia: -	Male and female Wistar rats (180-220 g).	-	↓ Food consumption; ↓ growth rate; ↓ water consumption; ↓ activity; ↓ grooming.	(Farhan *et al.*, 2014)

4.2 UCMS decreased plasma ACTH levels

Our data revealed that the UCMS protocol resulted in decreased plasma ACTH levels, with no changes in corticosterone or epinephrine concentrations (Figure 8-10). This was unexpected as previous UCMS studies largely showed increased plasma ACTH (Table 5: studies 1-3) and corticosterone levels (Table 5: studies 1; 3; 7; 8; 11-12; 18-19). This could be due to differences in stressor severity, frequency and duration as well as differing methods of euthanasia. Interestingly, studies primarily employing decapitation, with or without anesthesia or carbon dioxide asphyxiation noted differences in plasma corticosterone levels (Table 5: studies 1; 3; 7; 8; 11-12; 18-19). Some terminated the animals using an intraperitoneal injection of sodium pentobarbital and found no differences in plasma corticosterone, however they did observe alterations in urinary corticosterone levels (Bernberg *et al.*, 2009). This highlights the impact of varying methods of euthanasia on systemic concentrations of stress-related hormones.

As we suspected an injection of sodium pentobarbital may recruit neuroendocrine stress-response mechanisms, we instead measured plasma ACTH and corticosterone levels in blood that was drawn a week prior to the end of the UCMS protocol. However, it is likely that this blood draw was itself a stressful experience as it occurred in a room the rats were unfamiliar with, they were handled for a brief period of time by a trained professional the rats were not habituated to, and it is possible that the rats were aware of themselves losing their inhibitions. Activation of the stress response would have upregulated HPA axis and SNS activity, culminating in a surge of glucocorticoids and catecholamines into systemic circulation.

The lack of differences in corticosterone levels together with reduced ACTH concentrations may be representative of either hypersensitization of the adrenal cortex to ACTH or, enhanced negative feedback capabilities of corticosterone on the hypothalamus and pituitary gland. Because this is a unique finding compared to other UCMS protocols, further research is required to determine which of these hypotheses may be more plausible. However, a metabolic hypothesis of glucocorticoid function was proposed to explain the observation that

patients with stress-related psychiatric disorders often demonstrate moderately reduced or no changes in corticosterone levels (Yehuda and Seckl, 2011). This theory proposes that chronic stress exposure upregulates glucocorticoid receptor expression which sensitizes HPA axis negative feedback inhibition. Furthermore, impaired glucocorticoid catabolism may ensue, increasing the active period of cortisol. Thus, downstream glucocorticoid-mediated pathologies may still arise despite no changes in circulating plasma levels (Yehuda and Seckl, 2011). Contrastingly to depressed individuals, patients with PTSD typically present with reduced HPA axis activity. We thus speculate that the rats in our study may be exhibiting symptoms of PTSD rather than depression however further studies are required to confirm this. Lastly, we recommend follow-up studies also investigate GR expression in the hypothalamus and pituitary gland as well as the presence of morphological changes to adrenal tissue, such as hyperplasia or hypertrophy.

Corticosterone can also be measured in a variety of other sources e.g. saliva, urine, hair or faeces (Golbidi *et al.*, 2015). Although salivary glucocorticoids function as a marker of acute HPA axis activity, this method is beneficial because extracting saliva does not induce pain or discomfort in subjects. Furthermore, samples may be stored at room temperature for up to four weeks (Bozovic *et al.*, 2013). By contrast, hair glucocorticoid concentrations reflect long-term stress levels as human hair grows at a rate of approximately 1 cm/month (Golbidi *et al.*, 2015). The measurement of fecal corticosterone metabolites was also identified as an attractive non-invasive option for the long-term assessment of glucocorticoid levels (Thanos *et al*., 2009). Notably, acute stress responses and diurnal variations have limited influence on hair and fecal glucocorticoid measurements (Meyer *et al.*, 2014). Thus, we recommend assessing hair and fecal corticosterone levels at the beginning and end of the experimental procedure.

Taken together, our data highlights the variable nature of the UCMS model and the lack of a standardized approach. Moreover, it is likely that our current model is simulating PTSD instead of depression, although further studies are required to confirm this. We also conclude

that the model can be further refined in terms of the measurement of stress biomarkers. Here corticosterone levels should ideally be measured at multiple time-points and in different sources to gain a better understanding of glucocorticoid concentrations over the course of the entire experimental procedure and the choice of euthanasia be carefully considered.

4.3 Chronic stress impaired aortic vasodilation

Our findings show impaired endothelium-dependent dilation together with no observed differences in vasoconstriction (Figures 12-16) which is in agreement with most studies (Table 5: studies 7-9; 12-14). However, others have shown increased sensitivity to Phe-induced vasoconstriction (Neves *et al.*, 2009). The ability of ACh to induce vascular smooth muscle cell relaxation is heavily influenced by the integrity of the endothelium (Fuchgott and Zawadski, 1980). As no signs of endothelial damage were observed (Figure 11) and because of the essential vasodilatory properties of NO, impaired vasorelaxation is likely due to decreased NO bioavailability. This suggests that chronic stress may impair endothelial function by depleting NO levels. Reduced NO bioavailability could be a consequence of limited availability of L-arginine, eNOS uncoupling or inhibition as well as increased NO degradation (Malekmohammad *et al.*, 2019). Aside from the eNOS-inhibiting effects of glucocorticoids, the SNS induces drastic increases in heart rate and blood pressure, which may further impact the integrity of the endothelium and contribute towards endothelial dysfunction (Nickel *et al.*, 2009). However, we observed no differences in ET-1 which is a marker for endothelial damage (Figure 11) (Fox *et al.*, 2018). Glucocorticoid synthesis-inhibitors improve endothelium-dependent dilation by increasing NO levels (Broadley *et al.*, 2005, 2006). Glucocorticoids and catecholamines can further induce a dysregulated proinflammatory and prooxidative state which can potentiate endothelial dysfunction (Zielińska *et al.*, 2016; Burford *et al.*, 2017).

4.4 UCMS attenuated vascular SOD activity

Increased oxidative stress represents an important avenue for the development of endothelial dysfunction. A dysregulated oxidative state is often a result of increased free radical production or an impaired antioxidant defence system, such as SOD, GSH and catalase (Figure 17). We

noted decreased SOD activity and no significant differences in NOX activity following eight weeks of UCMS (Figure 17). This finding is supported by a number of recent studies (Table 5: studies 17-19). As NOX contributes to SO formation and SOD catalyzes the conversion of SO into hydrogen peroxide, blunted SOD activity suggests elevated vascular SO. Superoxide is a potent free radical and is directly implicated in the onset of endothelial dysfunction primarily via eNOS uncoupling (Santilli *et al.*, 2015; Incalza *et al.*, 2018). This occurs as a result of oxidation of tetrahydrobiopterin, an important eNOS cofactor. Following this, dysfunctional eNOS synthesizes less NO and this may explain our finding of impaired endothelium-dependent dilation. However, in the presence oxidative stress both increased and decreased eNOS activity were reported (Kamper *et al.*, 2009; Hicham *et al.*, 2018). This may be a compensatory mechanism whereby eNOS attempts to increase its activity to produce more NO in the hope of maintaining endothelium-dependent vasodilation (Isingrini *et al.*, 2012). Furthermore, others found no correlations between oxidative or inflammatory markers with blunted endothelial function (D'Audiffret *et al.*, 2010). Given that the pathological effects of stress on the body are heavily influenced by the manner in which stressors are perceived, and thus the degree of upregulation of stress-response mechanisms, this suggests that vascular dysfunction induced by the UCMS may not be a consequence of more common culprits, but may be due to mechanisms yet to be identified.

4.5 Rats were age-matched but not weight-matched

Although the rats in the beginning of our study were aged-matched, they were not weight-matched (Figure 5). This was unfortunate and only detected at the end of our experimental run. To determine whether this would have an impact on our biochemical measures (Figure 8-17), we consulted with a statistician (Prof Martin Kidd). Here we first checked to determine whether body weight correlated with any of our findings. We thereafter performed an analysis of covariance (with body weight as the covariate) to detect whether body weight affected our results. Of note, such analyses reported no correlations or interactions for body weight.

Although we acknowledge this as an unfortunate limitation of our study, the results of the statistical analyses corroborate our findings. In support, a number of studies found alterations in corticosterone concentrations as well as impaired endothelium-dependent dilation with no differences in body weight (Table 5: studies 7; 13-14). The Stress group in our study demonstrated an increased growth rate compared to Controls (Figure 6) that is opposite to Farhan *et al*. (2014) who attributed a reduced growth rate to the onset of depressive-like behaviours. However, because the Stress rats weighed less from the beginning of the experimental procedure, their increased growth rate likely occurred as there was more scope for them to grow compared to Controls, or because they displayed increased food intake at weeks 5, 6 and 8 (Figure 7).

4.6 Limitations

Despite our best efforts to improve upon our first experimental protocol, our second study is not without its own limitations. These include age-matched but not weight-matched experimental groups as well as the lack of standard behavioural assessments (reward test, splash test, novelty suppression test, immobility test). Despite this, we did assess coat condition at the end of each week. A score of 0 or 1 was assigned to the body part of each rat if said part was either clean or dirty. However, because this was assessed without an intervention (such as a spray of sucrose-water), grooming score more accurately reflected the cleanliness of the cage, as opposed to the rats' motivational behaviour. Control rats were also housed two-per-cage and thus their coats were often in better condition because they were able to groom each other.

We also measured the blood pressure and heart rate of each rat at the start, middle and end of the UCMS protocol. However, this measurement was flawed as it involved restraining the rats in a transparent Perspex tube. Restraining the rats in this manner is a stressful experience and thus our results did not accurately reflect blood pressures for the duration of the protocol. As we also experienced technical difficulties with the blood pressure apparatus these data were not included.

5. Conclusion

In summary, our results provide further support for the role of chronic stress in potentiating CVD. Following eight weeks of UCMS, we observed impaired endothelium-dependent dilation which is potentially a result of increased oxidative stress. Due to the damaging nature of this free radical, dysregulated SO concentrations can promote endothelial dysfunction via eNOS uncoupling and downregulated NO levels. This study highlights the importance of healthy coping mechanisms in reducing the onset of not only poor mental health, but cardiovascular derangements too. Thus, we propose a push for the integration of psychiatric therapies together with established cardiovascular risk-reducing behaviors. Education programs should focus especially on youth development within this context as adoption of healthy strategies from a younger age will ensure lasting changes to the state of stress in SA.

5.1 Future directions

We propose that future studies investigate the effects of UCMS stressors during the awake phase as rodents have evolved to respond to stressors during a state of consciousness. This will increase the translation of findings to the human context.

We further suggest that a “gold standard” for the UCMS protocol, i.e. in terms of stressor types, severity, frequency and duration should be established. Stress as a pathological stimulus is already an abstract and difficult-to-assess risk factor for disease progression, while its downstream negative effects are further confounded by the heterogenous manner whereby organisms perceive and subsequently adapt to such stressors. Limiting this variability should enhance the applicability and translation of results from animal models of chronic stress into the clinical context.

Future research work should also focus on identifying and confirming mechanisms of reduced NO bioavailability within the context of disrupted eNOS activity and regulators.

6. References

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Appendices

Appendix A

UNIVERSITEIT
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Approved with Stipulations

Date: 20 June 2018

PI Name: Mr Lucien Sher

Protocol #: 6311

Title: Investigating the effects of chronic stress on cardiovascular function

Dear Lucien Sher

The Investigating the effects of chronic stress on cardiovascular function submission was reviewed on 20 June 2018 by Research Ethics Committee: Animal Care and Use via committee review procedures and was approved on condition that the following stipulations are adhered to:

1. In the response to modifications, the applicant states that the aim of experiment 1 is to "establish the stress model" in their department and "to assess to what extent the rats are actually stressed". Considering this statement, the committee is of the believe that the planning of experiments 3 and 4 (which should now be 2 and 3) is premature. These experiments are dependent on a working stress model.
For this reason, only experiment 1 is approved at this time. Once this stress model is confirmed, the applicant must inform the committee (via submitting a progress report) so that experiments 2 & 3 can be reassessed.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number 6311 on any documents or correspondence with the REC: ACU concerning your research protocol.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

If you have any questions or need further help, please contact the REC: ACU secretariat at wabeukes@sun.ac.za or 021 808 9003.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

Appendix B

The protocol for the following ELISAs (Elabscience Biotechnology Inc, Houston TX) will be discussed concurrently due to identical methodology: ACTH (E-EL-R0048), corticosterone (E-EL-R0269), and E (E-EL-0045). The ET-1 kit (E-EL-R1458) was conducted in a slightly different manner and will thus be separately discussed.

Reagent preparation: ACTH (E-EL-R0048), corticosterone (E-EL-R0269) and E (E-EL-0045).

All reagents were brought to room temperature (~18-25°C) before use.

1. **Wash buffer (750 mL):** 30 mL of Concentrated Wash Buffer was diluted with 720 mL of deionized water.
2. **Standard working solution:** After centrifuging (Boeco M240, Hamburg, Germany) for 1 minute at 10, 000 *x g*, 1 mL of Reference Standard and Sample Diluent were combined to produce a 1, 000 pg/mL working solution. Serial dilutions were then prepared with the following dilution gradient: 1, 000, 500, 250, 125, 62.5, 31.25 and 0 pg/mL.
3. **Biotinylated Detection Ab working solution:** A 1x working solution was prepared with the Biotinylated Detection Ab Diluent (50 µL/well).
4. **Concentrated HRP Conjugate working solution:** A 1x working solution was prepared using the Concentrated HRP Conjugate Diluent (100 µL/well).

Assay procedure: ACTH (E-EL-R0048), corticosterone (E-EL-R0269) and E (E-EL-0045).

1. The varying concentrations of the Standard Working Solution were added in duplicate (50 µL/well). 50 µL of sample was then added to the remaining wells, also in duplicate (50 µL/well). Biotinylated Detection Ab working solution was then

immediately added to each well (50 µL/well). A sealer was used to cover the plate before it was then incubated at 37°C for 45 minutes.

2. 350 µL of Wash Buffer was then added to each well and allowed to soak for 1-2 minutes. This wash step was repeated 3 times.
3. HRP Conjugate working solution was then added (100 µL/well), the plate covered with a sealer and subsequently incubated for 30 minutes at 37°C.
4. The plate was then washed 5 times as conducted in step 2.
5. 80 µL of Substrate reagent was then added to each well before incubating the plate at 37°C for another 15 minutes.
6. Following the final incubation step, 50 µL of Stop Solution was pipetted into each well.
7. The optical density was then determined immediately using a micro-plate reader (EZ Read 400 Microplate reader, Biochrom, Holliston MA) set to 450 nm.

Reagent preparation: ET-1 kit (E-EL-R1458)

All reagents were brought to room temperature (~18-25°C) before use.

1. **Wash buffer (750 mL):** 30 mL of Concentrated Wash Buffer was diluted with 720 mL of deionized water.
2. **Standard working solution:** After centrifuging (Boeco M240, Hamburg, Germany) for 1 minute at 10 000 *x g*, 1 mL of Reference Standard and Sample Diluent were added to produce a working solution of 1000 pg/mL. Serial dilutions were then prepared with the following dilution gradient: 1000, 500, 250, 125, 62.5, 31.25 and 0 pg/mL.
3. **Biotinylated Detection Ab working solution:** A 1x working solution was prepared with the Biotinylated Detection Ab Diluent (50 µL/well).

4. **Concentrated HRP Conjugate working solution:** A 1x working solution was prepared using the Concentrated HRP Conjugate Diluent (100 µL/well).

Assay procedure: ET-1 kit (E-EL-R1458)

1. The varying concentrations of the Standard working solution was added in duplicate (100 µL/well). 50 µL of sample was then added to the remaining wells, also in duplicate (100 µL/well). The plate was then covered an incubated at 37°C for 90 minutes.
2. The solution was subsequently removed and 100 µL of Biotinylated Detection Ab working solution was added to each well before another incubation period of 1 hour.
3. 350 µL of Wash Buffer was then added to each well and allowed to soak for 1-2 minutes. This wash step was repeated 3 times.
4. HRP Conjugate working solution was then added (100 µL/well), the plate covered with a sealer and then it was incubated for 30 minutes at 37°C.
5. The plate was then washed 5 times as conducted in step 2.
6. 90 µL of Substrate reagent was then added to each well before incubating the plate at 37°C for another 15 minutes.
7. Following the final incubation step, 50 µL of Stop Solution was pipetted into each well.
8. The optical density was then determined immediately after with a micro-plate reader (EZ Read 400 Microplate reader, Biochrom, Holliston MA) set to 450 nm.

Appendix C

This appendix details the protocol used for the dissection of rat aortas and the assessment of endothelial function.

Reagent preparation

KH buffer, Phe and ACh was made fresh each morning according to the recipes detailed below.

1. **KH buffer:**

KH buffer was prepared from five individual buffers, using distilled water (dH_2O) (Table 6). These buffers were stored at 4°C for the entire experimental procedure.

Table 6: Individual buffers used to make KH buffer.

Buffer	Amount required
1. NaCl	279.00 g/ 2 L
2. $NaHCO_3$	83.60 g/ 2 L
3. KCl	17.60 g/ 1 L } **1 L total**
$KHPO_4$	8.10 g/ 1 L
4. $MgSO_4.7H_2O$	7.40 g/ 1 L } **1 L total**
Na_2SO_4	4.20 g/ 1 L
5. $CaCl_2.H_2O$	18.00 g/ 1 L

Buffer #1, #2, #3, #4 and glucose were added to 3 L of distilled water in a conical flask. Once the reagents completely dissolved, buffer #5 was added and the total volume was made up to 5 L with dH_2O. The prepared KH buffer was then filtered before use.

2. **0.9% Saline solution:** 9 g of NaCl was dissolved in 1 L of dH_2O.
3. **Phe stock solution (1 mM):** 0.002 g of Phe was dissolved in 10 mL of 0.9% saline.
4. **ACh:**
    - Stock A (10 mM) - Dissolve 0.0182 g of ACh in 10 mL of 0.9% saline.
    - Stock B (1 mM) – Add 1 mL of Stock A to 9 mL of 0.9% saline.
    - Stock C (100 µM) – Add 1 mL of Stock B to 9 mL of 0.9% saline.

Dissection procedure

Great care was taken to not stretch or otherwise damage the aorta during the dissection procedure:

1. Following the removal of the heart, the chest cavity was rinsed with approximately 5 mL of KH buffer.
2. Using a pair of tweezers, the aorta was gently pushed aside while the connective tissue between the aorta and vertebral column was dissected.
3. The aorta was then placed in a 100 mm petri dish containing ice-cold KH buffer.
4. All adventitia and connective tissue were subsequently removed using a magnifying glass. This step was carried out with care and efficiency to prevent damage to the endothelial cell layer and impaired vascular function.
5. Any blood clots within the vessel were removed before a 3 mm piece of tissue from the center of the vessel was carefully mounted onto two steel hooks. We also ensured that the tension on the rig was enough to keep the aortic ring in place. However, this was done carefully to prevent any unnecessary stretching of the tissue.
6. The ring was lowered into pre-warmed KH buffer (36.5-37°C).

Experimental protocol

The temperature of the organ bath was constantly measured and maintained between 36.5 and 37°C for the duration of the experiment:

1. The aortic ring was afforded 5 minutes to acclimatize the oxygenated KH buffer at a tension of approximately 0.2 g.
2. The tension was gradually increased to 1.5 g over the course of 30 minutes, with buffer changes at 10 and 20 minutes, respectively.
3. After 30 minutes, the total volume of the organ bath was set to 25 mL exactly. 2.5 µL of 1 mM stock Phe solution was then added to the organ bath to induce vasoconstriction. Once maximal contraction had been reached (plateau), 25 µL of stock A ACh solution was administered.
4. Once maximal vasodilation had been reached, the organ bath was rinsed 3 times with pre-warmed KH buffer and refilled to 25 mL.
5. Another stabilization period of 30 minutes was then done at a tension of 1.5 g, with washing steps at 10 and 20 minutes, respectively.
6. After 30 minutes, cumulative concentrations of Phe followed by cumulative concentrations of ACh was administered as follows:
    a. Phe (Total volume = 25 µL):
        i. 2.5 µL of stock
        ii. 5 µL of stock
        iii. 5 µL of stock
        iv. 7.5 µL of stock
        v. 5 µL of stock

b. ACh (Total volume = 301.8 µL)

   i. 7.5 µL of Stock C

   ii. 17.5 µL of Stock C

   iii. 42.5 µL of Stock C

   iv. 14.3 µL of Stock B

   v. 220 µL of Stock B

7. Once the experiment was finished, the length of the ring was measured and weighed before the organ bath was rinsed with boiled dH_2O.

Appendix D

This appendix describes protocols used for the SOD and NOX assays.

SOD

This assay measures the auto-oxidation of 6-hydroxydopamine at 490 nm for ~4 minutes (Ellerby and Bredesen, 2000).

Reagent preparation

- **Phosphate buffer:** 50 mM Na-Pi; 0.5% (v/v) Triton X-100; pH 7.5.
- **SOD assay buffer:** 50 mM $NaPO_4^-$ without Triton X-100, pH 7.4.
- **6-hydroxydopamine:** nitrogen purge 10 mL MilliQ water with 50 µL perchloric acid for 15 minutes. Use 10 mL of this and add 4 mg 6-hydroxydopamine to this solution. Wrap in foil and store on ice.
- **DETAPAC:** 0.4 mg in 10 mL of SOD assay buffer. Store at -20°C.

Tissue sample preparation

1. Approximately 100 mg of tissue was combined with 1 mL of phosphate buffer and homogenized using a Bullet Blender (Next Advance, Troy NY) (speed setting of 8) for five times one-minute periods, with a minute interval in between.
2. The homogenate was then sonicated (Misonixa ultrasonic liquid processor S-4000, Hielscher, Germany) (at amplitude setting of 10) for 10 seconds.
3. Sample was then centrifuged (Boeco M240, Hamburg, Germany) for 10 minutes at 15, 000 *x g* at 4°C.

Assay procedure

1. dH_2O acted as the blank and 12 µL was added in triplicate to a clear 96-well plate.
2. 12 µL of sample was then added in triplicate to all sample wells.

3. 15 µL of 6-HD was then added to each well.
4. Lastly, 170 µL of DETAPAC was pipetted into each well to initiate the reaction.
5. Absorbance was then measured for ~4 minutes at 490 nm using a plate reader (EZ Read 400 Microplate reader, Biochrom, Holliston MA).

NOX

A modified assay was used to determine the activity of NOX (Abid *et al*., 2007). In this reaction, the NADPH oxidase complex donates electrons to lucigenin. Lucigenin in turn emits photons of light which was measured over 20 minutes using a luminometer (Glomax-96 luminometer, Promega, Madison WI).

Reagent preparation

1. Prepare a modified assay buffer containing: 120 mM NaCl, 250 mM HEPES, 5.9 mM KCl, 11 mM glucose, 1.75 mM $CaCl_2(2H_2O)$, 0.5 mM EDTA, 100 µM NADH and 5 µM of lucigenin (pH 7.4).

Tissue sample preparation

1. Combine ~100 mg of tissue with 1 mL of phosphate buffer and homogenize using a Bullet Blender (Next Advance, Troy NY) (speed setting of 8) for five times one-minute periods, with a minute interval in between.
2. The homogenate was then sonicated (Misonixa ultrasonic liquid processor S-4000, Hielscher, Germany) (at amplitude setting of 10) for 10 seconds.
3. The sample was then centrifuged (Boeco M240, Hamburg, Germany) for 10 minutes at 15, 000 *x g* at 4°C.

Assay Procedure

1. 100 µL of sample and 100 uL of assay buffer were added (in duplicate) to each well of a 96-well plate.

2. The reaction was then measured every 5 minutes over a total period of 25 minutes at room temperature (Glomax-96 luminometer, Promega, Madison WI).

Appendix E

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Supplementary Data

This section includes all results obtained from the first experimental run – jointly completed by Lucien Sher and Lukas Olivier (during the first year of the respective MSc studies).

1. Baseline measurements

1.1 Body weight

At the end of each week, the body weight of each rat was measured (Figure 18) with the incremental weight gain expressed as a percentage of the rat's original weight (Figure 19). No significant differences in body weight or percentage weight gain was noted over the eight-week period.

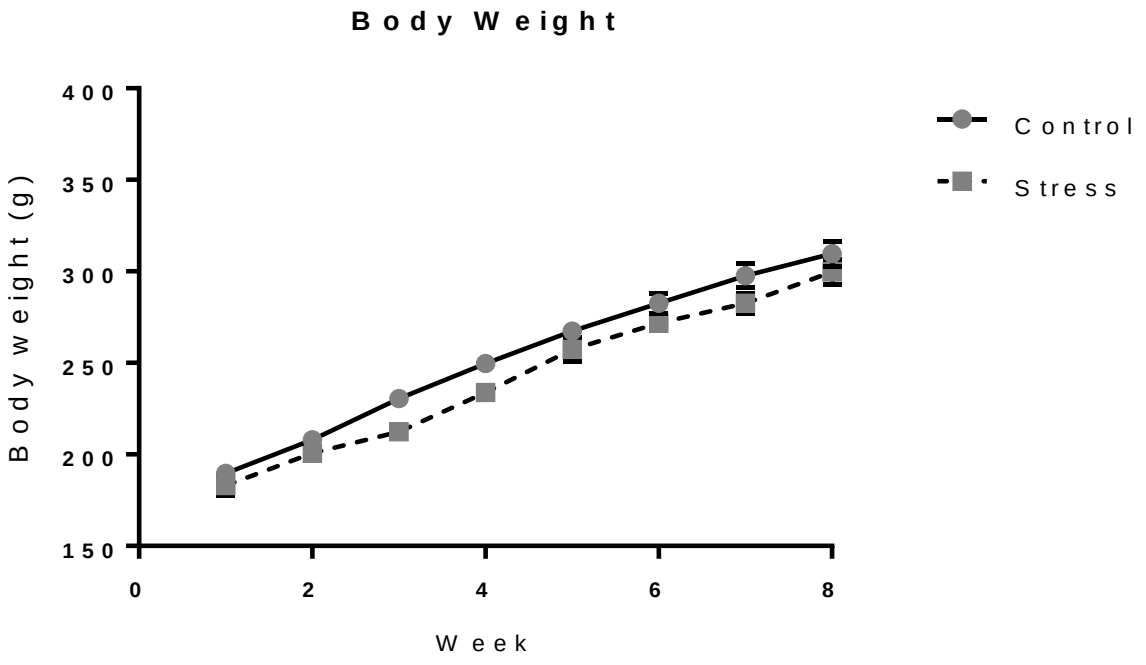

Figure 18: Weekly body weight measurements. Data displayed as mean ± SEM; statistical analyses: repeated measures, two-way ANOVA, Bonferroni post hoc; n = 9.

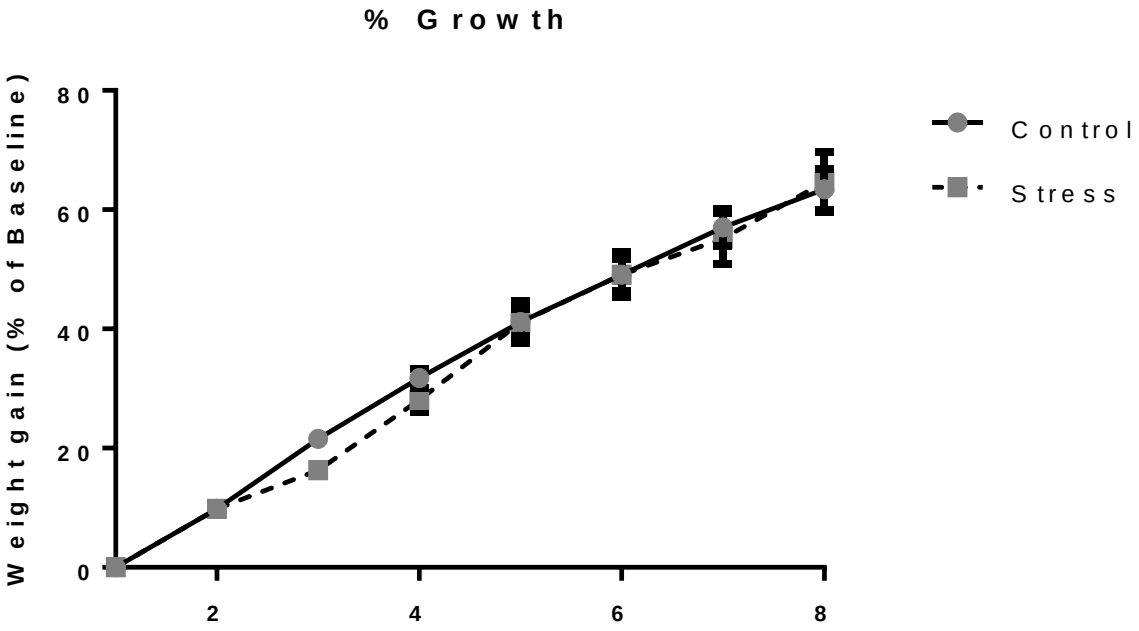

Figure 19: Percentage growth over the eight-week period. Data displayed as mean ± SEM; statistical analyses: repeated measures, two-way ANOVA, Bonferroni post hoc; n = 9.

1.2 Food Consumption

Food consumption was calculated by weighing the remaining pellets at the end of each week. Data shows that the Control group consumed more food than the Stress group during the first four weeks of the study; however, this was not significant (Figure 20).

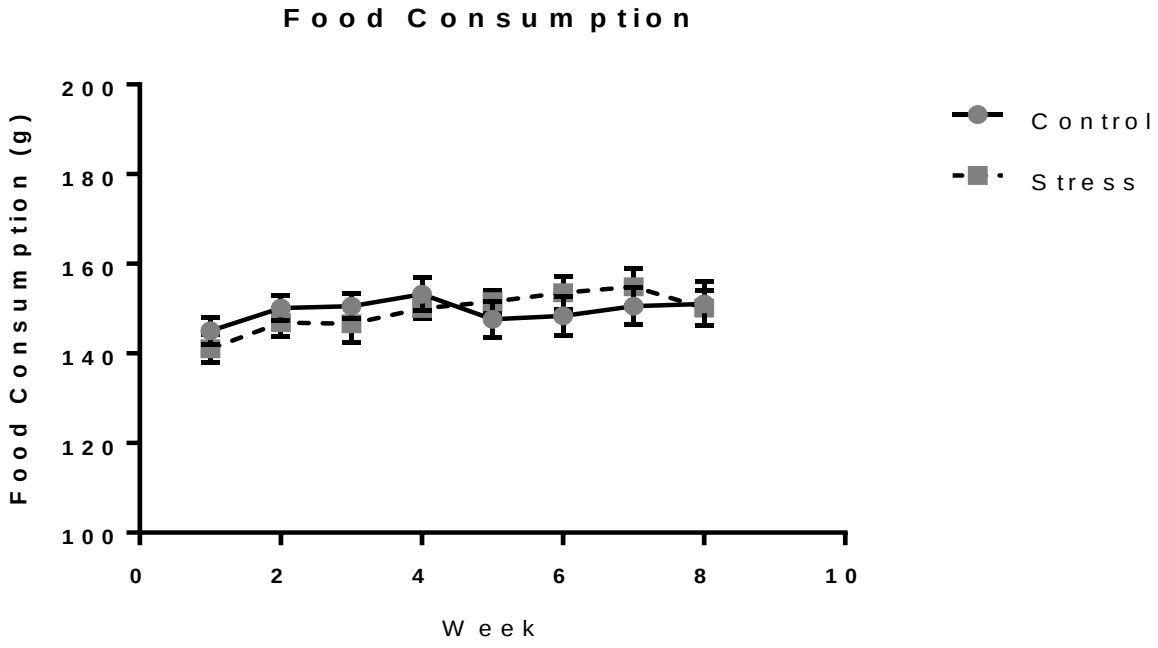

Figure 20: Amount of food consumed per week for each group. Data displayed as mean ± SEM; statistical analyses: repeated measures, two-way ANOVA, Bonferroni post hoc; n = 9.

2. ELISAs

ELISAs were obtained (Elabscience Biotechnology Inc, Houston TX) for the measurement of corticosterone (E-EL-R0269) and interleukin-6 (IL-6) (E-EL-R0015).

2.1 Corticosterone

Following the upregulation of the HPA axis, corticosterone is released from the zona fasciculata of the adrenal cortex. Corticosterone was measured in the plasma (Figure 21A) and in the left adrenal gland (Figure 21B) to discern circulatory glucocorticoids levels and to indirectly measure the production of corticosterone by the adrenal cortex. No significant differences in glucocorticoid levels were noted for either measurement.

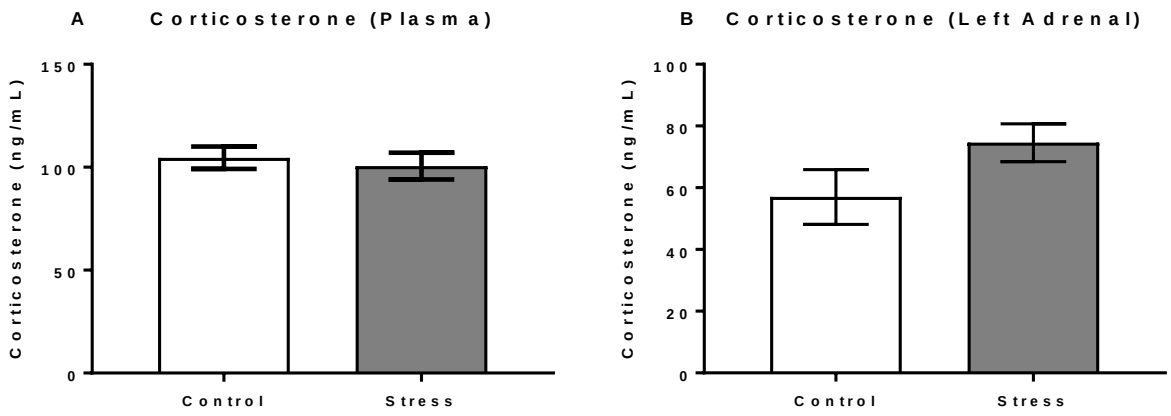

Figure 21: Corticosterone levels were assessed as a measure of HPA axis activity. For both figures (A and B), data displayed as mean ± SEM; statistical analyses: unpaired t-test; n = 9.

3. Oxidative stress

Oxidative stress is regarded as one of the primary mechanisms whereby stress-mediated molecular damage may occur (Incalza *et al.*, 2018).

Evidence also shows that the liver is particularly susceptible to an increased free radical load, thus also likely to show the first signs of oxidative damage compared to other tissues (Li *et al.*, 2015). Given that our initial protocol was relatively mild, and that the primary aim of this experiment was to successfully establish the UCMS model and induce a phenotype of chronic stress, we investigated the oxidative status of the liver.

Conjugated dienes and thiobarbituric acid reactive substances are early- and late-stage markers of free radical-induced lipid peroxidation. Although no significant differences were noted for this measurement (Figure 22A and B), we did observe a reduced antioxidant capacity of the Stress group when compared to Controls (Figure 22C). Glutathione is an essential anti-oxidant capable of preventing oxidative damage to a number of cellular structures and the ratio of reduced to oxidized glutathione is a common measure of oxidative stress (Masella *et*

al., 2005). However, we observed no differences in the ratio of reduced:oxidized glutathione levels in liver tissue (Figure 22D).

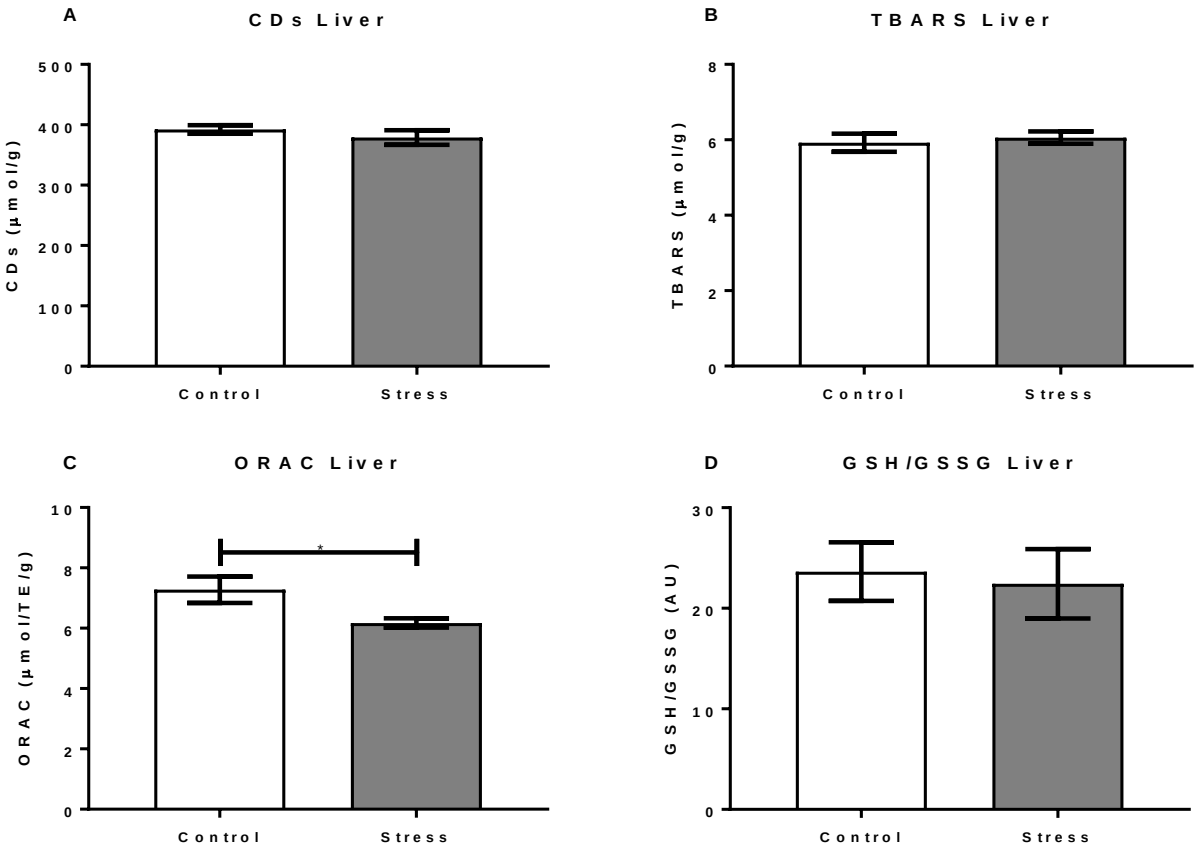

Figure 22: Analysis of various markers of oxidative stress in liver tissue. All data displayed as mean ± SEM; statistical analyses: unpaired t-test; n = 9. CDs - conjugated dienes; TBARS – thiobarbituric acid; ORAC – oxygen radical absorbance capacity; GSH – reduced glutathione; GSSG – oxidized glutathione.